STUDIES ON URINARY OESTROGENS

by

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I. GENERAL INTRODUCTION

For many years, ovariectomy has been known to cause cessation of oestrus and modification of secondary sex characteristics in animals. In 1913, Fellner observed that transient oestrous symptoms could be produced by injecting an ovariectomized animal with extracts of ovaries. These oestrous symptoms could be followed in the rat by observing histological changes in the vaginal epithelium and in 1923 this keratinization of the vaginal wall was used in the development by Allen and Doisy (1923) of a quantitative test for oestrogenic substances.

Four years later, using this method of bio-assay, Ascheim and Zondek (1927) discovered that extracts of human pregnancy urine possessed much greater oestrogenic activity than did ovarian extracts. This observation led to a closer examination of pregnancy urine and within two years the first crystalline oestrogenic substance, oestrone (oestra-1:3:5-triene-3-ol-17-one), was isolated from urine independently by two groups of workers - Doisy, Veler and Thayer (1929) and Butenandt (1929). In the following year,

oestriol/

oestriol (oestra-1:3:5-triene-3:16α:17β-triol) was isolated from the same source first by Marrian (1930) and soon after by Doisy, Thayer, Levin and Curtis (1930).

In 1933, Schwenk and Hildebrandt succeeded in reducing oestrone to an oestradiol (later shown to be oestradiol-17β (oestra-1:3:5-triene-3:17β-diol)) which was found to possess higher oestrogenic activity than either oestrone or oestriol. As a result of this finding, oestradiol-17β was adopted as the probable active principle secreted by the ovaries. Although this oestrogen was isolated from pregnant mares' urine (Wintersteiner, Schwenk and Whitman, 1935) and sows' ovaries (MacCorquodale, Thayer and Doisy, 1936) it was not until 1939 that Smith, Smith, Huffman, MacCorquodale, Thayer and Doisy isolated it from human pregnancy urine.

Between 1939 and 1953 no further metabolites of oestrogen metabolism were isolated from urine although a number of investigators reported the presence in various urine extracts of unknown substances whose chemical and physical properties suggested/

suggested that they might prove to be derivatives of known oestrogens (Pincus and Pearlman, 1943; Serchi, 1952; Zondek and Finkelstein, 1952; Migeon, 1953; Braunsberg, Stern and Swyer, 1954). Only one of these led to the actual isolation of an oestrogen metabolite, viz. 16-oxo-oestrone (oestra-1:3:5-triene-3-ol-16:17-dione) which Serchi (1953) obtained in crystalline form from the urine of nonpregnant women.

However, during the years from 1950-1954, two greatly improved chemical methods for the determination of oestrogens in urine were developed by Brown (1955b) and Bauld (1953). Both of these methods could produce relatively pure extracts on which the highly specific Kober reaction, as modified by Brown (1952) and Bauld, (1954) could be successfully used. Using his own method, Brown obtained convincing evidence for a fourth Kober chromogen in acid-hydrolysed human pregnancy urine, while Bauld, using the second of the above mentioned procedures, detected a similar Kober chromogen in his 'oestriol' fraction from the urine of a nonpregnant woman.

This fourth Kober chromogen was isolated from human pregnancy urine by Marrian and Bauld (1954)/

(1954) and identified as 16-epioestriol (oestra-1:3:5-triene-3:16β:17β-triol). Although they did not establish with certainty that this substance was not an artifact of their isolation procedure, Section II of this thesis will deal with that possibility.

It seems probable, also, that enzymic hydrolysis of urinary conjugates will lead to the detection of oestrogen metabolites which are sensitive to the rather harsh process of boiling the urine with acid. This thesis, for instance, describes the isolation of a fifth Kober chromogen which is largely destroyed by hot acid hydrolysis and which, without the aid of enzymic hydrolysis, would have been extremely difficult to isolate. In addition, a sixth Kober chromogen has been detected in extracts of enzyme-hydrolysed urine by my colleague, Mr K.H.Loke (personal communication) in extremely small amounts.

With the increased availability of radioactively labelled oestrogens, metabolic studies have indicated the excretion of oestrogen derivatives other than those already isolated. For instance, Levitz, Spitzer and Twombly (1956) claim/

claim to have detected labelled 16-oxooestradiol-17β (oestra-1:3:5-triene-3:17β-diol-16-one) in urine of two postmenopausal women after the administration of oestradiol-17β-16^{14}C; Slaunwhite and Sandberg (1956) report the presence of 16-oxooestrone in the urine of one woman to whom they had administered oestrone-16 ^{14}C during the luteal phase of the menstrual cycle; more recently, Kraychy and Gallagher (1957) have published a preliminary report of their isolation of 2-methoxyoestrone from urine after the administration of oestradiol-17β-16 ^{14}C.

It is hoped that these techniques will lead to the isolation of other oestrogen metabolites and so establish at least some of the reactions which these hormones undergo in the body.

II. SOME OBSERVATIONS ON THE OCCURRENCE OF 16-EPIOESTRIOL IN URINE

1. Introduction

In their isolation of 16-epioestriol (oestra-1:3:5-triene-3:16β:17β-triol) from the urine of pregnant women, Marrian and Bauld (1955) did not exclude the possibility that this substance was artifactually produced during the isolation procedure by epimerization of the 16-hydroxyl group of oestriol (oestra-1:3:5-triene-3:16α:17β-triol). The yield of 16-epioestriol was low (0.1 mg./l.) and since the urine had been hydrolysed by hot acid and the extracts were subsequently in contact with aqueous alkali, such a conversion seemed quite probable. Obviously, this question had to be settled before proceeding to further study of the occurrence of 16-epioestriol in urine or in other body fluids.

Samples of authentic oestriol were therefore boiled with acid and treated with aqueous alkali under conditions similar to those existing during the/

the processing of urine. The resultant products were then analysed by partition chromatography. A method was then devised for the rough determination of 16-epioestriol in urine. Using this method, a comparison was made of yields of 16-epioestriol from pregnancy urine after hot acid hydrolysis with those obtained after hydrolysis with β-glucuronidase.

Having established 16-epioestriol as a true constituent of pregnancy urine, a preliminary study of its excretion during the follicular and luteal phases of the menstrual cycle was undertaken.

2. General Methods Employed

(a) Acid hydrolysis of urine

This was effected by heating the urine to boiling, adding 15 vols.% 10 N-HCl and continuing the boiling for one hour.

(b) Preparation of β-glucuronidase from Patella vulgata (Dodgson and Spencer, 1953).

The Patella vulgata or common limpets were collected in large batches, removed from their shells and the black visceral humps retained. These viscera were twice homogenized in ice-cold acetone/

acetone as quickly as possible. The resulting 'whole' enzyme contained in the acetone powder was dried in air and passed through a sieve to remove portions of muscular debris and, at the same time, to render the powder homogeneous.

For some experiments, a water-soluble enzyme was prepared by extracting the whole enzyme with water and reprecipitating the water-soluble glucuronidase from this solution by the addition of eight times its volume of acetone. The resultant powder was dried.

The enzyme, whether 'whole' or water-soluble, was assayed according to the method of Fishman, Springer and Brunetti (1948) using phenolphthalein glucuronide as substrate. Briefly, this method involved incubating the enzyme with phenolphthalein glucuronide at pH 4.7 in 0.1M-acetate buffer at 37°C. for one hour. The reaction mixture was then buffered to pH 10.4 with glycine/NaOH/NaCl buffer and the intensity of the pink colour read at 540 mμ.

The 'whole' enzyme had an activity varying from 300,000-1,300,000 Fishman units/g., the variation being apparently partly due to the season/

season of year in which the limpets were collected. The water-soluble preparation contained 1,300,000-2,000,000 Fishman units/g. of powder.

In addition to β-glucuronidase activity, both of these enzyme preparations contained an unknown amount of various sulphatases.

(c) Enzymic hydrolysis of urine

For enzymic hydrolysis, urine was acidified to pH 4.7 (Cohen, 1951) with glacial acetic acid and buffered to that pH by the addition of one tenth of its volume of M-acetate buffer; 1,000,000 Fishman units of glucuronidase/l. urine were added and the mixture incubated at 37°C. for two days. When 'whole' enzyme was used, the powder was mixed in a small volume of water which was added to the urine.

(d) The development of a method for the estimation of 16-epioestriol in urine

The method developed for the rough determination of 16-epioestriol in urine was a modification of that of Bauld (1953) for the determination of oestriol.

Preliminary experiments with oestriol and 16-epioestriol showed that in the benzene-water partition of the Bauld method, about 95% of the oestriol present is in the aqueous phase, while there is rather less than 60% of the 16-epioestriol, because/

because of its relatively lower 'polarity'. However, the proportion of 16-epioestriol in the aqueous phase could be raised to about 85% by doubling the proportion of water used in the partition. About 8% of any oestradiol-17β present was found to pass into the aqueous phase but did not interfere in the subsequent separation of the 16-epioestriol fraction on the chromatograms.

It seemed likely that in the determination of 16-epioestriol in pregnancy urines the relatively large amounts of oestriol present might interfere in the separation of the 16-epi-oestriol fractions in the chromatograms. However, this potential difficulty could be overcome by washing the final ether extract obtained before chromatography with a pH 12.4 phosphate buffer. By this means 70-90% of the oestriol present could be removed with little loss of 16-epi-oestriol.

The complete procedure for estimation of 16-epioestriol in urine was as follows:-

For urines from normal menstruating women, determinations were carried out on one half of the 24 hour specimen, and for urines of pregnant

women, on one tenth of the 24 hour specimen diluted to 500 ml. with water. After hydrolysis with acid or enzyme, the urine was extracted four times with about one quarter of its volume of ether. The combined ether extract was washed once with 100 ml. of pH 10.5 NaOH/$NaHCO_3$ buffer, shaken with 25 ml. of 2 N-NaOH and, after the addition of 100 ml. of 8.5% (w/v) $NaHCO_3$, reshaken and the aqueous layer discarded. After further washing, once with 25 ml. of 8.5% (w/v) $NaHCO_3$ and three times with 12 ml. of water, the extract was evaporated to dryness.

This residue was dissolved in 1.5 ml. of ethanol and quantitatively transferred to a separating funnel with 25 ml. of benzene. The benzene solution was extracted twice with 50 ml. and twice with 25 ml. of water. After the addition of 15 ml. of 10 N-NaOH, the combined aqueous extracts were boiled under reflux for 30 minutes, cooled, neutralized to phenolphthalein by passing CO_2 into the solution and extracted four times with 50 ml. portions of ether.

With samples of urine from pregnant women, this ether extract was washed twice with 40 ml. portions of pH 12.4 phosphate buffer, the buffer washing/

washing back-extracted once with 40 ml. of ether and the combined ether extracts washed twice with 24 ml. portions of water and evaporated to dryness.

With samples of urine from the menstrual cycle, the extract was not washed with buffer, but once with 12 ml. of 8.5% (w/v) $NaHCO_3$ and twice with 6 ml. portions of water before being evaporated to dryness.

The residue was chromatographed using the system 70% (v/v) methanol/ethylene dichloride as described in the following sections.

(e) Partition chromatography of products

Partition chromatography was based on the method described by Bauld (1955). The two phases were equilibrated overnight. A 5 ml. portion of the stationary phase was thoroughly mixed into 5 g. of Celite. Sufficient mobile phase was then added to prepare a slurry which was packed into a 'micro' column, 1 cm. in diameter, to a height of 10 cm. so that mobile phase could percolate through at a rate of 10-12 ml./hour. 'Channelling', due to uneven packing, was prevented by rotating the column during packing.

The/

The residues to be analysed were dissolved in mobile phase and a known aliquot pipetted on to the column. As soon as this solution had completely entered the column, elution with mobile phase was started. Fractions of eluate of the required volume were collected and subjected to a Kober reaction.

(f) The modified Kober reaction

The Kober reaction as modified by Brown (1952) and Bauld (1954) was used. The reagent employed, which was that used by Bauld for the estimation of oestriol, was prepared as follows.

Hydroquinone (40 g.) was dissolved in 2 l. of 76% H_2SO_4 containing 40 mg. of quinone and 20 mg. of $NaNO_3$.

For the Kober reaction, 50 mg. of hydroquinone were added to each fraction before evaporating to dryness. (In systems using ethylene dichloride in the mobile phase, particular care had to be taken to remove every trace of this solvent since it gives a brown colour in the Kober reaction). Oestriol reagent (2.6 ml.) was added to each residue and the mixture heated in a vigorously boiling water bath/

bath for 20 minutes, during which time the tubes were twice shaken to ensure complete solution of the residue. After five minutes' cooling in water, a further 50 mg. of hydroquinone followed by 0.7 ml. of water were added to each tube, and the latter were heated for 15 minutes - again, in a boiling water bath and with adequate shaking. The solutions were immediately cooled and the optical densities of the solutions measured in 10 mm. glass cells at wavelengths of 480, 512.5 and 545 mμ. By subtracting the average of the readings at 480 and 545 mμ from the optical density at 512.5 mμ, (Allen, 1950) a corrected optical density proportional to the amount of oestriol present was obtained.

3. <u>The Treatment of Oestriol with Acid</u>

Three 3 ml. portions of an ethanolic solution of oestriol containing 50 μg./ml. were added, respectively, to three 500 ml. portions of water. After heating the solutions to boiling under reflux, 75 ml. of 10 N-HCl were added to each and the boiling continued for periods of 1, 2 and 4 hours, respectively. An untreated/

FIG.1. TYPICAL ELUTION PATTERNS OF OESTRIOL AND 16-EPIOESTRIOL IN SYSTEM 70% (V/V) METHANOL / ETHYLENE DICHLORIDE.

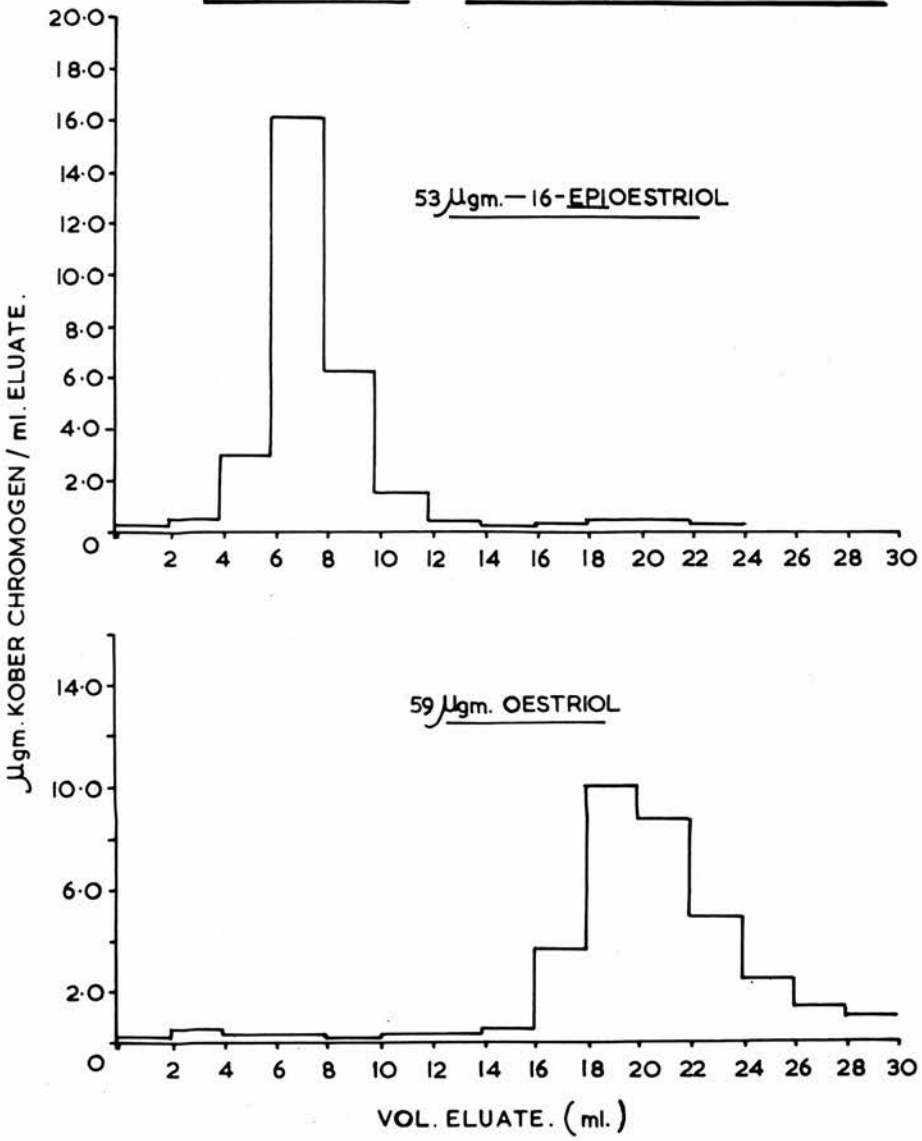
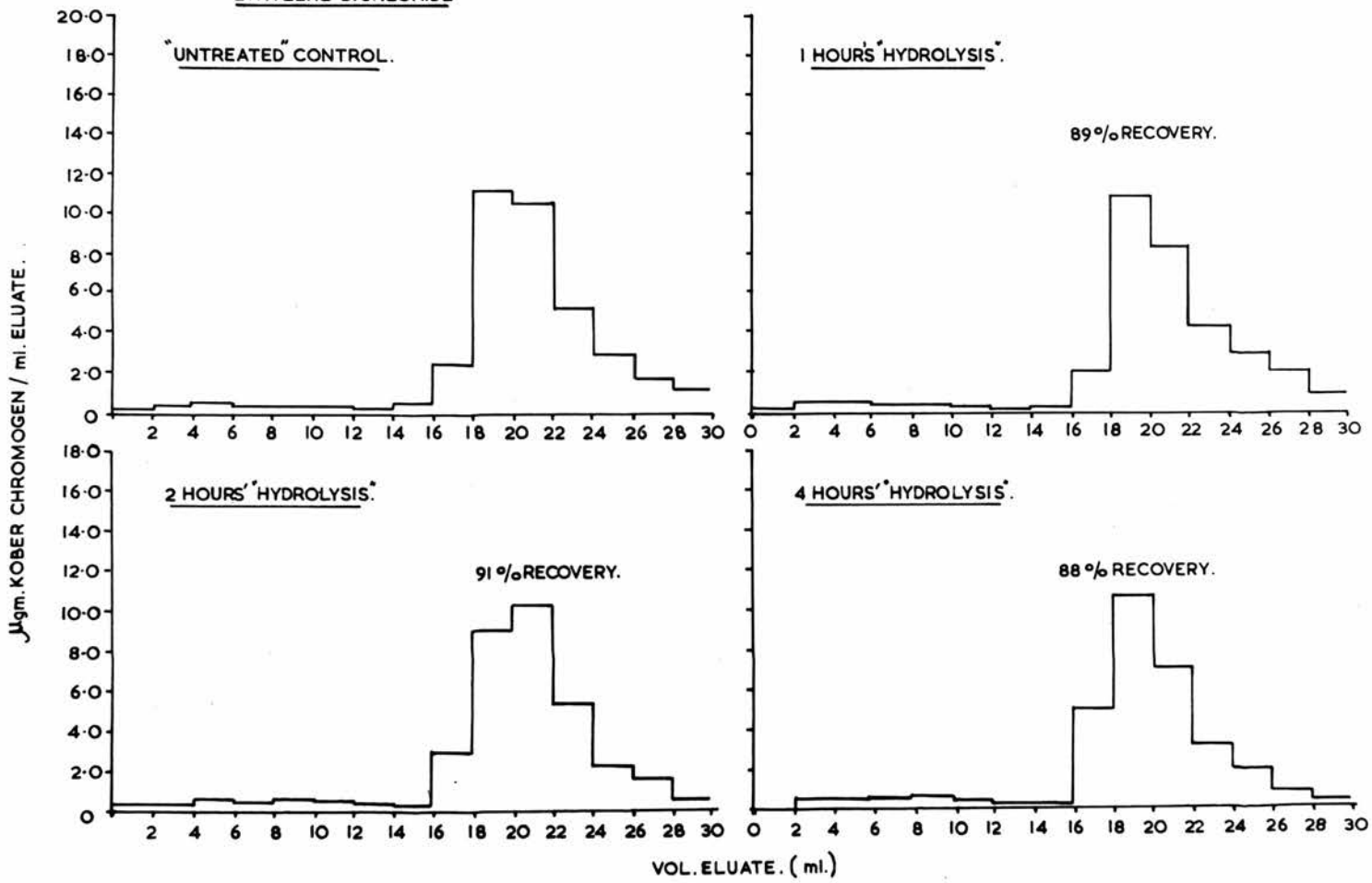


FIG. 2. HOT ACID TREATMENT OF OESTRIOL. – PARTITION CHROMATOGRAMS IN SYSTEM 70% (V/V) METHANOL / ETHYLENE DICHLORIDE



untreated control solution was prepared by adding 3 ml. of the solution of oestriol to 575 ml. of water.

Each of the four solutions was extracted once with 150 ml. and three times with 125 ml. of ether. The combined ether extract of each solution was washed three times with 50 ml. portions of 8.5% (w/v) $NaHCO_3$, three times with 50 ml. portions of water and evaporated to dryness.

One half of each product was analysed by partition chromatography on a 'micro' column with the system 70% (v/v) methanol/ethylene dichloride. In this system, 16-epioestriol is eluted in the fraction 4-12 ml. and oestriol in the fraction 16-26 ml. (Fig. 1). The recoveries of oestriol in the three 'acid hydrolyses' compared with that in the control were 89, 91 and 88% respectively, and in no case was there evidence for a Kober chromogen less 'polar' than oestriol (Fig. 2).

It can be concluded from this that oestriol is not epimerized to any extent by boiling in aqueous solution with 15 vols.% of 10 N-HCl for periods of up to 4 hours.

4. /

4. Treatment of Oestriol with Aqueous Alkali

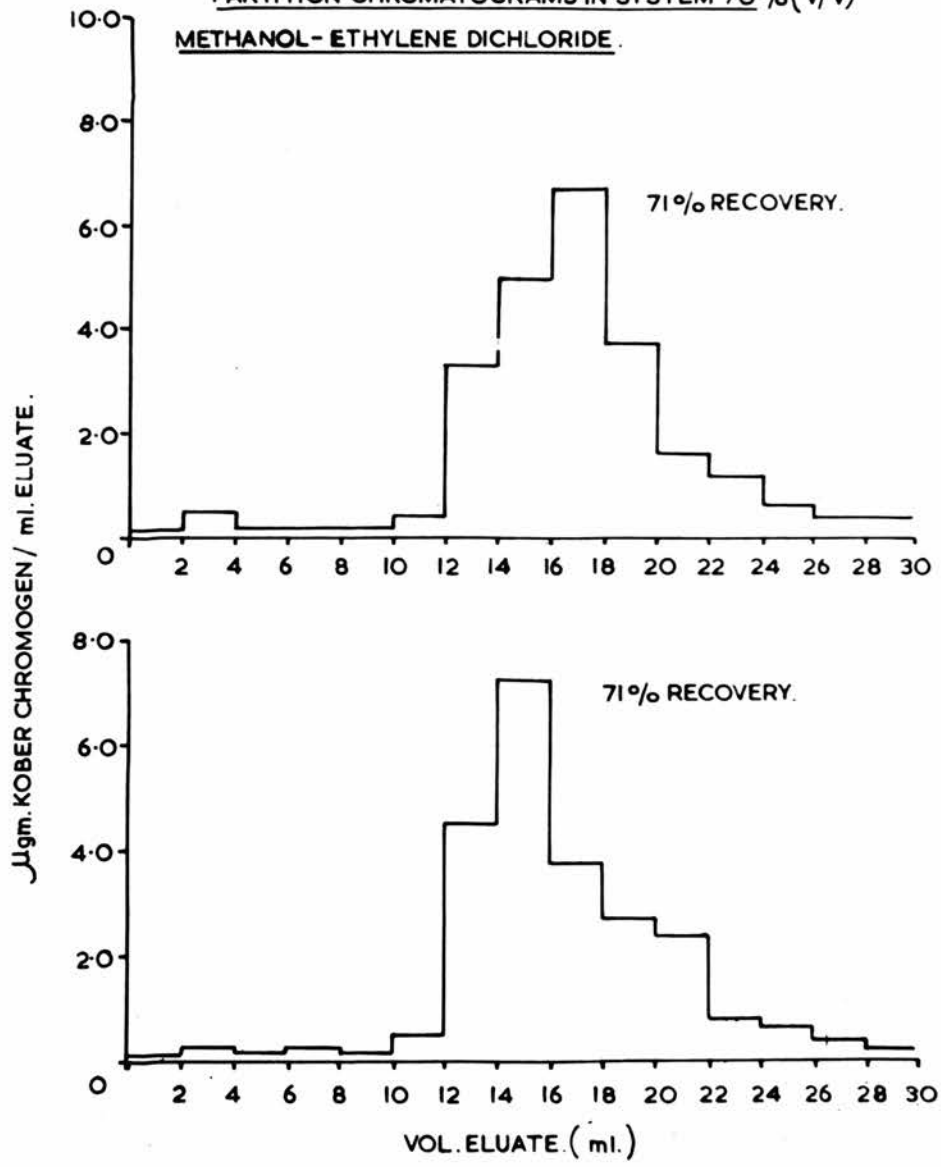
To investigate the possibility that epimerization of oestriol to 16-epioestriol might occur through exposure to aqueous alkali under the conditions used in the quantitative procedure of Bauld (1953) or in the procedure for the isolation of 16-epioestriol as used by Marrian and Bauld (1955), the following experiment was carried out.

Each of two solutions containing 75 µg. of oestriol in 575 ml. of water was extracted once with 150 ml. and three times with 125 ml. of ether. Each of the combined ether extracts was washed once with 100 ml. of $NaHCO_3$/NaOH buffer, pH 10.5, shaken with 25 ml. of 2 N-NaOH and, after the addition of 100 ml. of 8.5% (w/v) $NaHCO_3$, reshaken and the aqueous phase discarded. The ether solutions were further washed once with 25 ml. of 8.5% (w/v) $NaHCO_3$ and three times with 12 ml. of water and evaporated to dryness.

The residues were each dissolved in 150 ml. of water, and, after the addition of 15 ml. of 10 N-NaOH, boiled under reflux for 30 minutes. CO_2 was passed into the solutions until they were just acid to phenolphthalein. The solutions were/

FIG.3. ALKALI TREATMENT OF OESTRIOL.

PARTITION CHROMATOGRAMS IN SYSTEM 70% (V/V) METHANOL - ETHYLENE DICHLORIDE.



were then extracted four times with 50 ml. of ether and the combined ether extracts washed once with 12 ml. of 8.5% (w/v) $NaHCO_3$, twice with 6 ml. of water and evaporated to dryness.

An aliquot of each residue was chromatographed on a micro column with the system 70% (v/v) methanol/ethylene dichloride. The recovery of oestriol in both cases was only 71% but no evidence was found for a Kober chromogen less 'polar' than oestriol (Fig. 3).

It was therefore concluded that treatment with aqueous alkali, as employed in the procedure for the purification of urine extracts, does not result in the formation of significant amounts of 16-epioestriol by the epimerization of oestriol.

Since the alkali treatment involved in the isolation procedure used by Marrian and Bauld was considerably milder and of shorter duration than that described above, it can be assumed with reasonable safety that no significant amount of 16-epioestriol would be produced from oestriol during the isolation process.

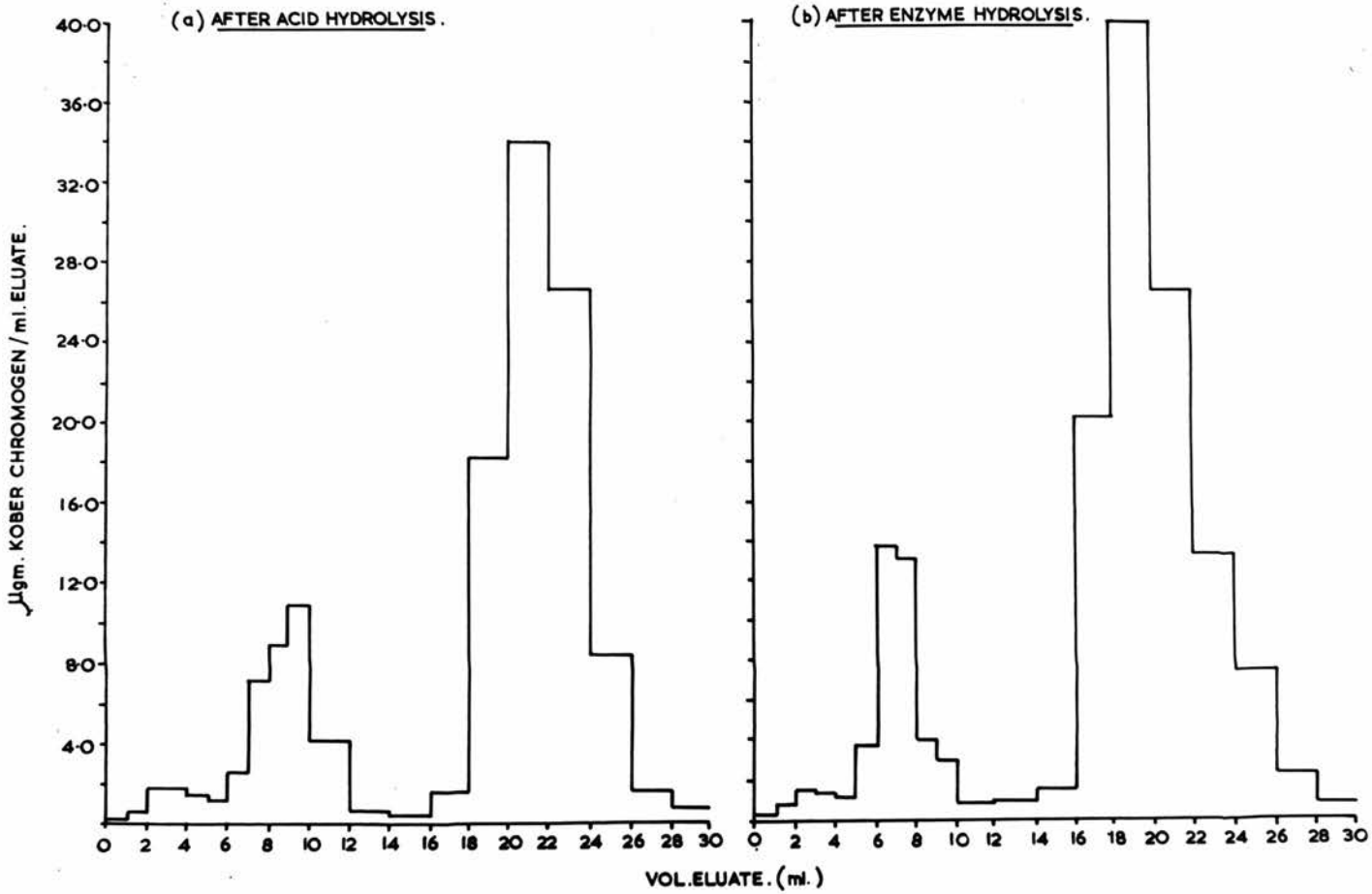
5. /

5. A Comparison of the Yields of 16-Epioestriol from Pregnancy Urine after Hot Acid Hydrolysis with those obtained after Hydrolysis by β-Glucuronidase

Although the results described in Section II,3 indicated that prolonged boiling of aqueous solutions of oestriol with 15 vols.% of 10 N-HCl did not cause any detectable formation of 16-epi-oestriol, it was still possible that oestriol conjugates in urine might undergo slight epimerization during hot acid hydrolysis. It was therefore decided to compare the yield of 16-epioestriol obtained from pregnancy urine after hot acid hydrolysis with that obtained after incubation with a β-glucuronidase preparation from Patella vulgata.

Experiments were carried out on four different 24 hour urine specimens collected during late pregnancy. The specimens were diluted to 2500 ml. Two 250 ml. portions of this were removed, further diluted to 500 ml. and hydrolysed with hot acid in the usual way. Two further 250 ml. samples were hydrolysed under the usual conditions by incubating with 385 mg. of a water-soluble β-glucuronidase from Patella /

FIG.4. YIELDS OF 16-EPIOESTRIOL FROM PREGNANCY URINE. ($\frac{1}{10}$ × 24 hrs SAMPLE.).– PARTITION CHROMATOGRAMS IN SYSTEM 70% METHANOL – ETHYLENE DICHLORIDE.



Patella vulgata containing 1,500,000 units of activity/g. Each incubation mixture was finally diluted to 500 ml. before extraction.

The '16-*epi*oestriol' fraction was separated from each sample of hydrolysed urine by the method described in Section II.2d. Such fractions were analysed by partition chromatography in the system 70% (v/v) methanol/ethylene dichloride and Kober chromogens were determined on successive 1 ml. or 2 ml. portions of the eluate. Good separation of the 16-*epi*oestriol fraction was obtained (Fig. 4) and, although no claim is made that the whole procedure is quantitatively satisfactory, when applied to urines of pregnancy, it is believed to provide a reliable rough method of assessment which is of value for comparative purposes. It must, however, be added that this method is not specific for 16-*epi*oestriol since 17-*epi*oestriol (oestra-1:3:5-triene-3:16α:17α-triol) (Prelog, Ruzicka and Wieland, 1945) behaves very similarly to 16-*epi*oestriol in this chromatographic system.

The results of these estimations are shown in Table 1.

Table 1 /

Table 1. Comparison of yields of 16-epioestriol from pregnancy urine after hydrolysis with acid with those after hydrolysis with β-glucuronidase (Patella vulgata).

Urine specimen	16-epioestriol/24 hr. urine (μg.)	
	After acid hydrolysis	After enzymic hydrolysis
1	458, 442	445, 418
2	572, 572	352, 397
3	814, 788	628, 530
4	320, 327	425, 406

The fact that in each case enzymic hydrolysis yielded 16-epioestriol in amounts which were of the same order as those obtained after hot acid hydrolysis, disposes of the possibility that 16-epioestriol is formed by epimerization of oestriol conjugates during hot acid hydrolysis. In three of the four cases, rather higher amounts of 16-epioestriol were detected after acid hydrolysis than after enzymic hydrolysis. This may in part be explained by losses due to the rather troublesome emulsions which occur during ether extraction of enzyme -hydrolysed urine.

6. /

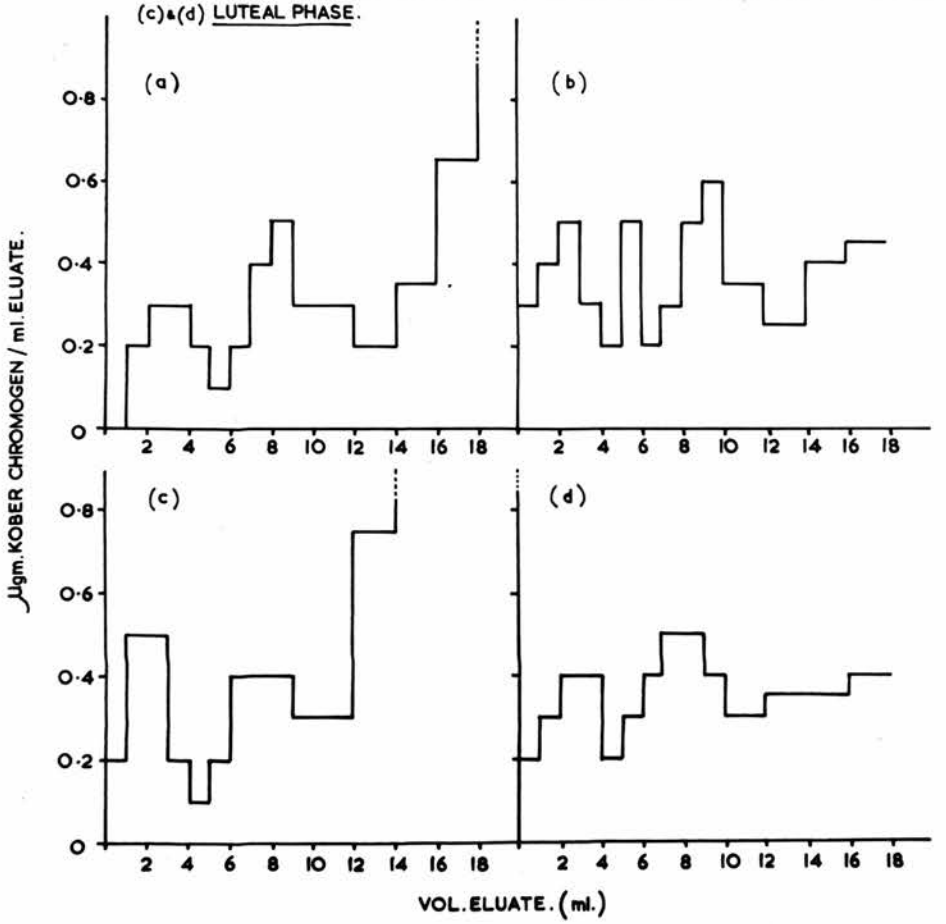
6. Preliminary Attempts to Detect 16-Epioestriol in Urines Excreted during the Follicular and Luteal Phases of the Menstrual Cycle.

To investigate whether or not 16-epioestriol is excreted in the urine during the menstrual cycle, six 24 hour specimens of urine were collected from two normal women. Three of these samples were excreted a few days before the expected time of ovulation, during the follicular phase of oestrogen secretion, and three about midway during the second half of the cycle during the luteal phase of oestrogen secretion. According to Brown (1955a), maximum excretion of oestrogen is expected at those stages in the cycle.

Each urine specimen was divided into two equal portions which were acid hydrolysed for one hour, and extracted as previously described (Section II.2). The final fractions were chromatographed on a micro column in the system 70% (v/v) methanol/ethylene dichloride and Kober chromogens were determined on 1 ml. or 2 ml. portions of the eluate.

Because such a large portion of the 24 hour sample was used in the determination, the background colour in the Kober reaction was much more marked/

FIG.5. PARTITION CHROMATOGRAMS (IN SYSTEM 70°/₀ (V/V) METHANOL / ETHYLENE DICHLORIDE) OF EXTRACTS OBTAINED FROM MENSTRUAL CYCLE URINES. (a) & (b) FOLLICULAR PHASE (c) & (d) LUTEAL PHASE.



marked and separation of 16-epioestriol much less clear-cut than in the case of extracts from pregnancy urine. Typical representative elution patterns are shown in Fig. 5. In each case, a Kober chromogen was eluted in the fraction between 6-12 ml. but the elution patterns were so confused by non-specific chromogens eluted near the solvent front that virtually no quantitative significance could be attached to these results and it seemed advisable to collect more evidence before deciding whether this chromogen eluted between 6 and 12 ml. was actually 16-epioestriol.

7. Identification of the '16-epioestriol-like' Kober Chromogen excreted during the Menstrual Cycle

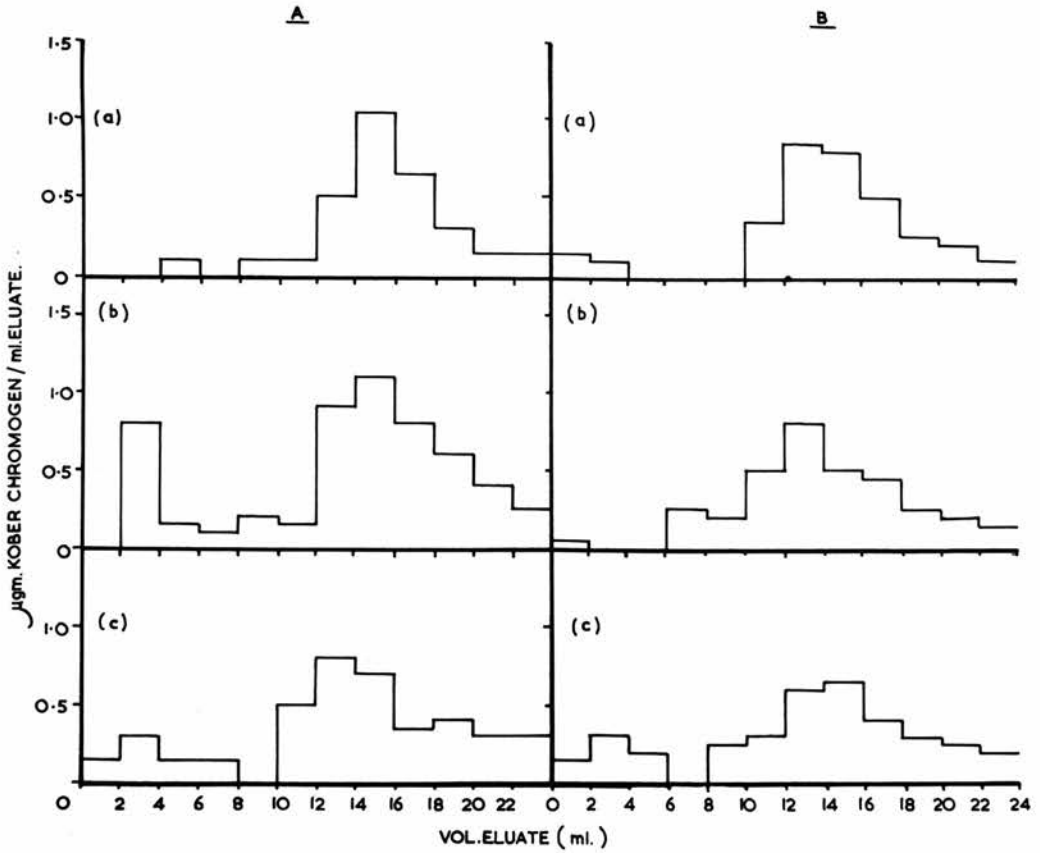
To obtain further evidence on the identity of this '16-epioestriol-like' Kober chromogen, fourteen and ten 24 hour urine specimens were collected during the follicular and luteal phases, respectively, from four different normal young women. Each sample was separately hydrolysed with acid, extracted and fractionated by the methods previously described, and finally chromatographed/

FIG.6. CHROMATOGRAPHIC IDENTIFICATION OF '16 EPI OESTRIOL-LIKE' FRACTIONS (E.L.F.) FROM MENSTRUAL CYCLE URINES.

A.-70% (V/V) METHANOL/BENZENE-ETHYLENE DICHLORIDE. (3:1 BY VOL.)

B.-50% (V/V) ETHANOL/n-HEXANE-CHLOROFORM. (3:1 BY VOL.)

(a) AUTHENTIC 16 EPI OESTRIOL. (b) E.L.F. FROM FOLLICULAR PHASE URINE. (c) E.L.F. FROM LUTEAL PHASE URINE.



chromatographed on a micro column with the system 70% (v/v) methanol/ethylene dichloride. The fractions eluted from the columns between 6 and 12 ml. from the fourteen follicular phase specimens were combined in one 'pool' and those from the luteal phase specimens combined in another. Each 'pool' was then divided into three equal portions.

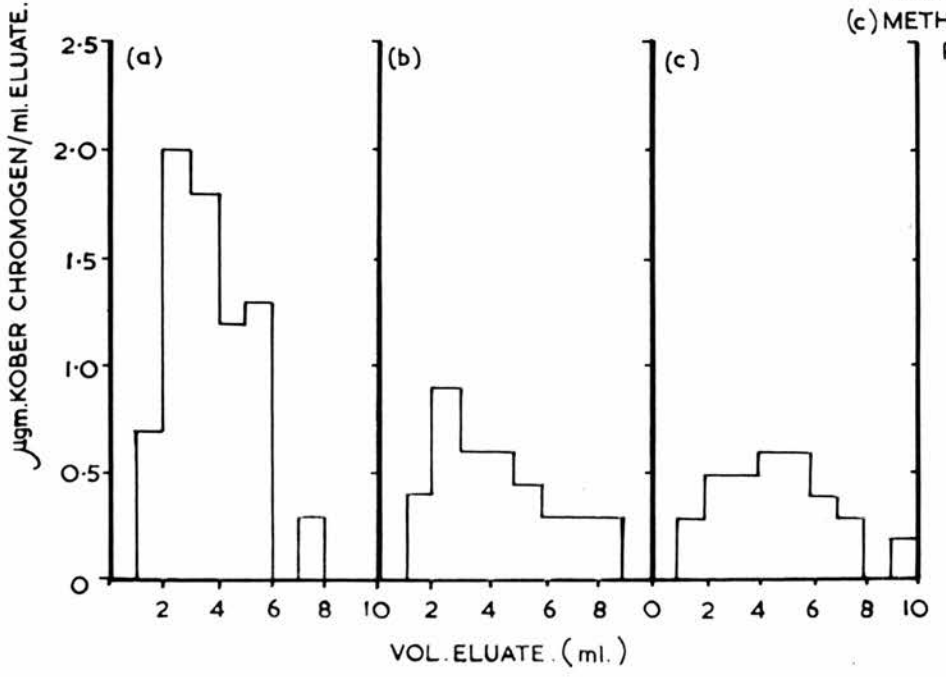
One portion from each pool was chromatographed on a micro column with the system 70% (v/v) methanol/benzene-ethylene dichloride (3:1 by vol.) while second portions were similarly chromatographed with the different system 50% (v/v) ethanol/n-hexane-chloroform (3:1 by vol.). For comparison authentic 16-epioestriol was chromatographed in each of these systems. The results (Fig. 6) showed that in both these solvent systems the chromatographic behaviour of the '16-epioestriol-like' Kober chromogens closely resembled that of authentic 16-epioestriol.

The remaining portion of each pool was then methylated as follows: The material was dissolved in 50 ml. of 0.4 N-NaOH and, after warming to 37°C., 1 ml. of methyl sulphate (redistilled) was added, the mixture shaken and maintained/

FIG.7. CHROMATOGRAPHIC IDENTIFICATION OF '16 EPIOESTRIOL-LIKE' FRACTIONS (E.L.F.) FROM MENSTRUAL CYCLE URINES.

[70%(V/V) METHANOL/BENZENE – n-HEXANE. (1:1 BY VOL.)]

(a) METHYLATED 16 EPIOESTRIOL.
(b) METHYLATED E.L.F. FROM FOLLICULAR PHASE URINE.
(c) METHYLATED E.L.F. FROM LUTEAL PHASE URINE.



maintained at 37°C. for 30 minutes. 5 N-NaOH (2 ml.) and a further 1 ml. of methyl sulphate were then added, and after shaking for a few minutes, the mixture was maintained at 37°C. for a further 45 minutes. After cooling, the mixture was extracted once with 100 ml. of ether and the extract washed four times with 25 ml. portions of water and evaporated to dryness. For comparison, 25 µg. of authentic 16-epioestriol was methylated under identical conditions.

Each of the methylated products was then chromatographed with the system 70% (v/v) methanol/benzene:n-hexane (1:1 by vol.). The results (Fig. 7) showed that in this solvent system the chromatographic behaviour of the methylated urinary '16-epioestriol-like' Kober chromogens closely resembled that of methylated 16-epioestriol.

8. Further Evidence for the Presence of 16-Epioestriol in Extracts of Urines excreted during the Follicular and Luteal Phases of the Menstrual Cycle

Some time after the experiments already described were completed, 24 hour samples of follicular and luteal phase urines were collected from/

from eight young normally-menstruating women. These samples were hydrolysed by enzyme and extracted by a method rather different from the one used in the preliminary search for 16-epi-oestriol in menstrual cycle urines. This method was not claimed to be even semi-quantitative and the urines were actually processed for the purpose of examining the Kober chromogen content of the ketonic fraction. However, the non-ketonic fractions were chromatographed and in the majority of these extracts, a Kober chromogen was detected whose elution closely resembled that of 16-epioestriol. Again, amounts were small and in a few samples the amount was negligible.

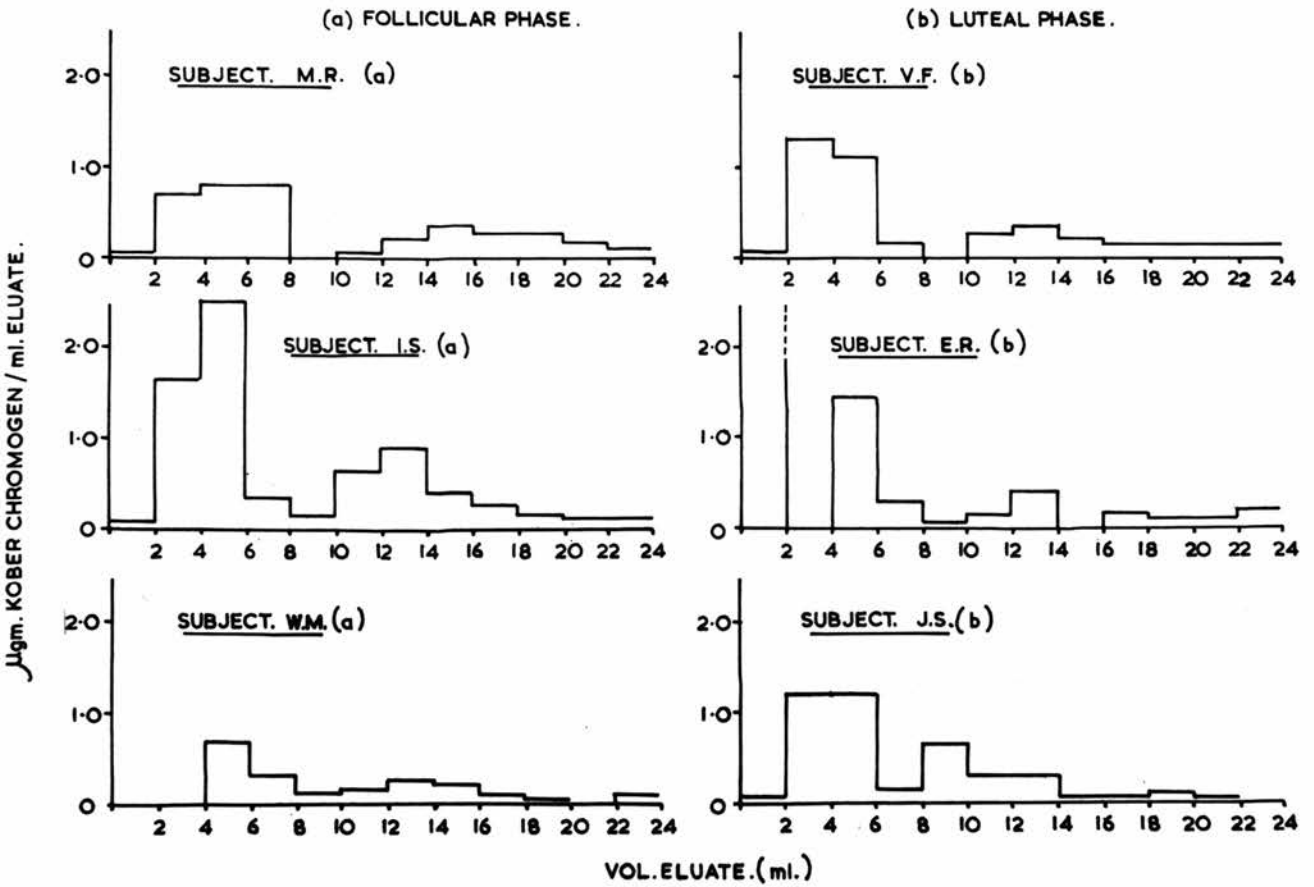
9. Outline of the Methods of Hydrolysis, Extraction and Chromatography

Since the exact experimental details of most of this method will be described in a later section of this thesis,(Section III,2) only a brief outline of that part will be given here.

The complete 24 hour sample of urine was hydrolysed for two days with 'whole' limpet enzyme.

The mixture was extracted with ether, washed/

FIG.8. REPRESENTATIVE PARTITION CHROMATOGRAMS OF THE NON KETONIC FRACTIONS OF MENSTRUAL CYCLE URINES IN SYSTEM 70%(V/V). METHANOL / BENZENE – ETHYLENE DICHLORIDE.(3:1 BY VOL.)



washed with $NaHCO_3$ and chilled before extracting with chilled N-NaOH. The phenolic fraction extracted by alkali was made just acid with chilled 5 N-H_2SO_4, then extracted into ether. The ether extract was washed with $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated to dryness.

The phenolic extract was then divided into ketonic and non-ketonic fractions by subjecting it to a Girard reaction at room temperature for 17 hours. The non-ketonic fraction was washed once with 10 ml. of $NaHCO_3$/NaOH buffer pH 10.5, shaken with 5 ml. of 2 N-NaOH and, after the addition of 20 ml. of 8.5% (w/v) $NaHCO_3$, reshaken and the aqueous layer discarded. After further washing, once with 10 ml. of 8.5% (w/v) $NaHCO_3$ and twice with 10 ml. of water, the extract was dried over Na_2SO_4 and evaporated to dryness.

This non-ketonic fraction was chromatographed with the system 70% (v/v) methanol/benzene-ethylene dichloride (3:1 by vol.). Fig. 8 shows partition chromatograms for six non-ketonic fractions, which provide an idea of the variation in the amount of '16-epioestriol-like/

like' material eluted in the fractions from 10-20 ml.

In themselves, these results are far from conclusive, but when it is taken into account that the method of hydrolysis and fractionation is markedly different from that previously described, they appear to supply additional evidence for the excretion of 16-epioestriol during the follicular and luteal phases of the menstrual cycle.

10. Discussion

The evidence presented in this section indicates that oestriol does not give rise to chromatographically detectable amounts of 16-epioestriol either by boiling with HCl for periods of up to 4 hours or by aqueous alkali treatment as employed in the fractionation of urinary extracts. It follows that, in the alkali fractionation involved in the isolation procedure of Marrian and Bauld (1955), no significant amount of 16 epioestriol would be formed by epimerization of oestriol. Also, it has been shown that yields of 16-epioestriol from/

from pregnancy urine are of the same order whether the urine is hydrolysed by boiling with acid or by incubating it with β-glucuronidase. It therefore can be concluded with reasonable certainty that 16-epioestriol is a true constituent of pregnancy urine. The quantities of 16-epioestriol in pregnancy urine were found to vary from 0.35-0.80 mg./24 hour sample, whereas Marrian and Bauld isolated it in amounts equivalent to only 0.10 mg./litre of urine. This discrepancy is possibly due to losses during crystallization and also, perhaps, to incomplete extraction of 16-epioestriol into the 'strong phenolic' fraction, effected by extracting an ether extract of pregnancy urine with 0.1 N-NaOH.

Evidence has also been presented to indicate the probable occurrence of small amounts (1 μg./24 hours) of a '16-epioestriol-like' Kober chromogen in menstrual cycle urines excreted during the follicular and luteal phases. The chromatographic behaviour of this Kober chromogen resembles that of authentic 16-epioestriol in three different partition systems. In addition, methylation of this Kober chromogen yields a product which resembles methylated 16-epioestriol in chromatographic behaviour.

Furthermore/

Furthermore, a similar Kober chromogen was detected in the majority of non-ketonic phenolic fractions which were prepared by a very different method from menstrual cycle urines of the follicular and luteal phases.

Although the amounts detected were very small, neither method is claimed to be quantitative and the true amount of 16-epioestriol present may be considerably higher than indicated. On the other hand, it must be remembered that the method is not specific for 16-epioestriol and it is quite possible that small amounts of 17-epioestriol may be present in the '16-epioestriol' fractions.

III. /

III. THE ISOLATION OF 16α-HYDROXYOESTRONE FROM THE URINE OF PREGNANT WOMEN

1. Introduction.

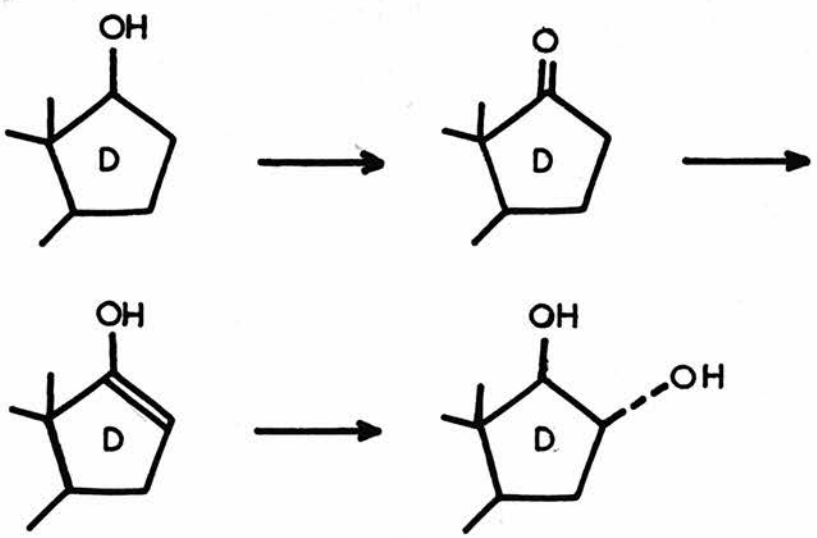
Since the isolation of oestrone (oestra-1:3:5-triene-3-ol-17-one), oestradiol-17β (oestra-1:3:5-triene-3:17β-diol) and oestriol (oestra-1:3:5-triene-3:16α:17β-triol), numerous attempts have been made to study the metabolic relationships of these three compounds. Administration of each hormone and subsequent search for metabolites in urine have established beyond any reasonable doubt that oestradiol-17β and oestrone are readily interconvertible and that either of them may be changed, apparently irreversibly, into oestriol (Pearlman, 1948). More recently, Pearlman, Pearlman and Rakoff (1954) confirmed these observations by detecting deuterated oestrone, oestradiol-17β and oestriol in the urine of pregnant women to whom they had administered 6.7 D_2-oestrone acetate. Also, in 1955, Beer and Gallagher studied the excretion of radioactive compounds after the administration of oestrone-16-^{14}C and oestradiol-17β-16-^{14}C to

women/

women. After treatment with labelled oestradiol-17β, radioactive oestrone and oestriol were excreted in the urine. The excretion pattern of radioactive metabolites after administration of oestrone-16-^{14}C was so similar to that observed after oestradiol-17β-16-^{14}C administration, that this evidence was taken to confirm the ready interconversion of these two hormones.

A number of compounds have been suggested as possible intermediates in the production of oestriol from either oestrone or oestradiol-17β. For instance, Marrian (1939) postulated that oestrone, produced by dehydrogenation of oestradiol-17β, might be rearranged to its enolic form which, on subsequent hydration could give rise to oestriol.

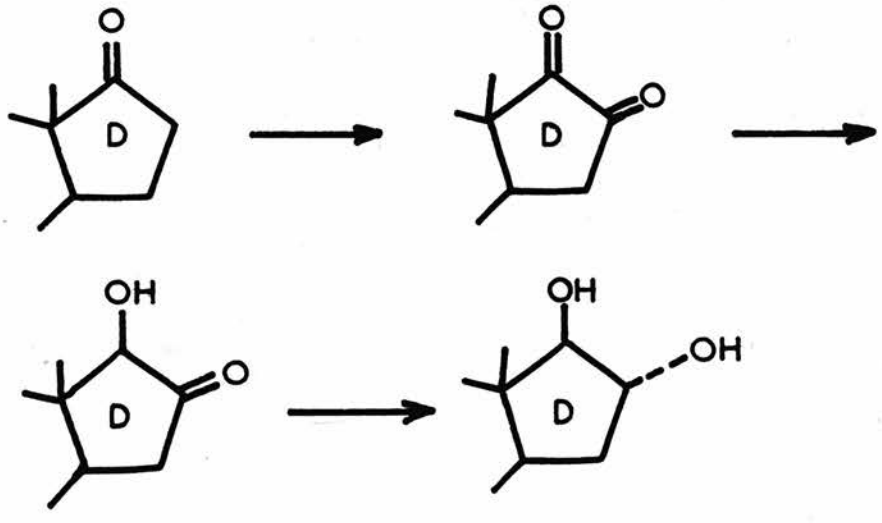
viz.



The first indication of the presence of some such intermediate in urine came from the observation by Smith and Smith (1941) that higher oestrogenic activity was obtained from urines which had been hydrolysed by zinc and hydrochloric acid than was obtained from urines hydrolysed by acid alone. They suggested that this increase might be due partly to the reduction of some inactive catabolic product to an active oestrogen. However, closer examination of their experimental procedure reveals that the conditions they chose for simple acid hydrolysis were far from optimal. They boiled urine with 15 vols.% of concentrated hydrochloric acid for only ten minutes, whereas Stevenson and Marrian (1947), Marrian (1948), Van Bruggen (1948), Stimmel (1949) and Engel (1950) all agree that boiling under those conditions must be continued for at least forty minutes in order to obtain maximal hydrolysis of the conjugated oestrogens in human pregnancy urine. In face of this overwhelming evidence that Smith and Smith (1941) were employing sub-maximal conditions for acid hydrolysis, it is thought that the bulk of the increase in activity after three hours' boiling with/

with zinc and hydrochloric acid, could be accounted for by more complete hydrolysis of the conjugated oestrogens and by reduction of oestrone to the more active oestradiol-17β.

In 1943, Pincus and Pearlman referred in a review article to their detection in extracts of human pregnancy urine of an oestrogen which was ketonic and contained an hydroxyl group in addition to the usual phenolic one. No experimental details were published. Four years later, Huffman and Grollman (1947) suggested that this substance might be 16-oxooestradiol-17β (oestra-1:3:5-triene-3:17-diol-16-one) since after a study of the biological activities of a number of oestrogen derivatives, they postulated the following mechanism for the conversion of oestrone to oestriol.



i.e. Oestrone is first oxidized to 16-oxooestrone (oestra-1:3:5-triene-3-ol-16-17-dione) and then stepwise reduced to oestriol by way of 16-oxo-oestradiol-17β.

Some support for this series of reactions was obtained from the observation by Stimmel, Grollman and Huffman (1950) that injection of 16-oxooestrone and of 16-oxooestradiol-17β caused the excretion of increased amounts of oestriol in urine.

Zondek and Finkelstein (1952) found that from certain urines there was a ratio of 5:1 between the oestrone fraction measured fluorimetrically and that measured biologically; they suggested that this discrepancy was due to some oestrogen metabolite of low biological activity which fluoresced as strongly as oestrone. In the same year, Serchi (1952) obtained evidence for two strongly phenolic unknown substances in extracts of urine from normal women and in the following year isolated 16-oxooestrone from the same source (Serchi, 1953).

In 1953, Migeon detected in extracts of acid hydrolysed urine from normal males and females and from six subjects suffering from hyperactivity/

hyperactivity of the adrenal glands, a sulphuric acid fluorogen whose 'polarity' was intermediate between that of oestradiol-17β and oestriol. Observing that this fluorogen had a partition coefficient similar to 16-oxooestradiol-17β in one of the solvent systems which he used in countercurrent distribution, he added the latter substance to urine and subjected it to the usual hydrolysis and fractionation but was unable to recover it. He therefore concluded that this unknown fluorogen was unlikely to be 16-oxooestradiol-17β. In one urine, a second fluorogen with a slightly different partition coefficient in the system used was detected. No attempt has yet been made to verify the possibility, but it seems likely that one of these fluorogens might be 16-epioestriol (oestra-1:3:5-triene-3:16β:17β-triol).

With the evidence that 16-epioestriol is a true constituent of pregnancy urine and is also very likely excreted during the menstrual cycle, interest in the metabolism and interconversion of the known oestrogens has been restimulated. Since both oestriol and 16-epioestriol are excreted, the most obvious precursor of both of these substances would seem to be 16-oxooestradiol-17β. Consequently, an attempt was made to detect this substance in human pregnancy urine.

2. General Methods Used

(a) Acid hydrolysis
(b) Preparation of β-glucuronidase
(c) Enzyme hydrolysis

(a), (b) and (c) were effected as described in Section II.2. In large-scale work, the whole enzyme was used and was added to the urine in two equal portions, one at the beginning of the incubation, the second 24 hours later.

(d) Method of extraction

Details of the method used in the preliminary detection of the fifth Kober chromogen (KC-5) and in studying certain aspects of its stability will be described in the appropriate section of a later part of this thesis. It will suffice at this point to say that, as certain properties of KC-5 became known, it was considered advisable to modify that preliminary method in order to increase the yield of KC-5 from urine. The final method used in large-scale work and in the preliminary search for KC-5 in menstrual cycle urines was as follows.

In large-scale work,600 g. of NaCl were dissolved in each 4 litre batch of enzyme-hydrolysed urine in order to facilitate the subsequent extraction of oestrogens into ether and to help prevent the formation of stable emulsions during ether extraction. The urine was then extracted once with an equal volume of ether. In small-scale work, no salt was added and the hydrolysed urine was extracted four times with one quarter or three times with one third of its volume of ether. The ether extract was washed once with one tenth of its volume of 5% (w/v) $NaHCO_3$ and then extracted twice with one eighth of its volume of chilled N-NaOH. (In small-scale work, the ether extract,itself, was also thoroughly chilled). The alkaline extract was partially neutralized by chilled 5 N-H_2SO_4 and brought to pH 9.0-9.3 with CO_2. The whole procedure of extraction and neutralization was carried out as quickly as possible.

The neutralized solution was re-extracted twice with an equal volume of ether, the extract washed twice with one quarter of its volume of water, dried over Na_2SO_4 and evaporated to dryness. This was the 'phenolic' fraction.

(e) /

(e) Girard reaction

In large-scale work, the phenolic fractions were collected until 100 litres of urine had been processed. These extracts were combined, treated with 50 ml. of ethanol, 10 ml. of glacial acetic acid and 5 g. of Girard's reagent T (trimethylammonium hydrazide chloride). The reaction was allowed to proceed at room temperature for approximately seventeen hours, after which the mixture was thoroughly chilled and 90% of the acid neutralized by NaOH in 500 ml. of a solution which also contained 50 g. of NaCl to help prevent emulsions in the subsequent ether extractions. The pH of this mixture was confirmed to be 7.0.

In work involving the extraction of separate 24 hour samples of urine, the phenolic fraction was treated with 2 ml. of ethanol, 2 ml. of glacial acetic acid and 100 mg. of Girard's reagent T. The neutralizing alkali and NaCl were added in 50 ml. of solution.

The neutralized mixture from each of these reactions was then washed once with four fifths and three times with two fifths of its volume of ether in order to remove non-ketonic material. The remaining aqueous ketonic fraction was acidified/

acidified with either 10 N-HCl or 50% (v/v) H_2SO_4 and left to stand at room temperature for one hour.

To obtain the ketonic phenolic fraction, this acidified solution was extracted three times with four fifths of its volume of ether in small-scale work, and four times with two fifths of its volume of ether in large-scale work. The combined ether extract was washed three times with one fifth of its volume of 8.5% (w/v) $NaHCO_3$, twice with one fifth of its volume of water, dried over Na_2SO_4 and evaporated to dryness.

(f) Partition chromatography

Partition chromatography was based on the technique described by Bauld (1955). Chromatography in micro columns was carried out as reported in Section II,2.

In isolation work, however, a larger column, 2 cm. in diameter, was used. Celite (120 g.) was treated with 120 ml. of stationary phase. Sufficient mobile phase was added to prepare a slurry, all of which was packed evenly on to the column with maximum pressure. Such a column/

column was used to separate KC-5 from oestrone in the phenolic ketonic fraction from 100 litres of urine. Since this fraction was bulky and only slightly soluble in the mobile phase generally used, it had to be introduced on to the column in the following way.

The ketonic phenolic fraction was dissolved in 3 ml. of stationary phase and quantitatively transferred, with the aid of a further 2 ml. of stationary phase, to a small beaker containing 5 g. of Celite. The solution was thoroughly stirred into the Celite. A total of 40 ml. of mobile phase was then used to complete the transfer of any remaining traces of the urinary fraction, prepare a slurry and pack it quantitatively on to the top of the column. Thirty 40 ml. fractions of eluate were collected automatically. The rate of percolation of mobile phase was generally about 45 ml./hour.

(g) Kober reaction

The method of Brown (1952) as modified by Bauld (1954) was employed, Bauld's 'oestriol' reagent being used. Details are given in Section II,2.

(h) /

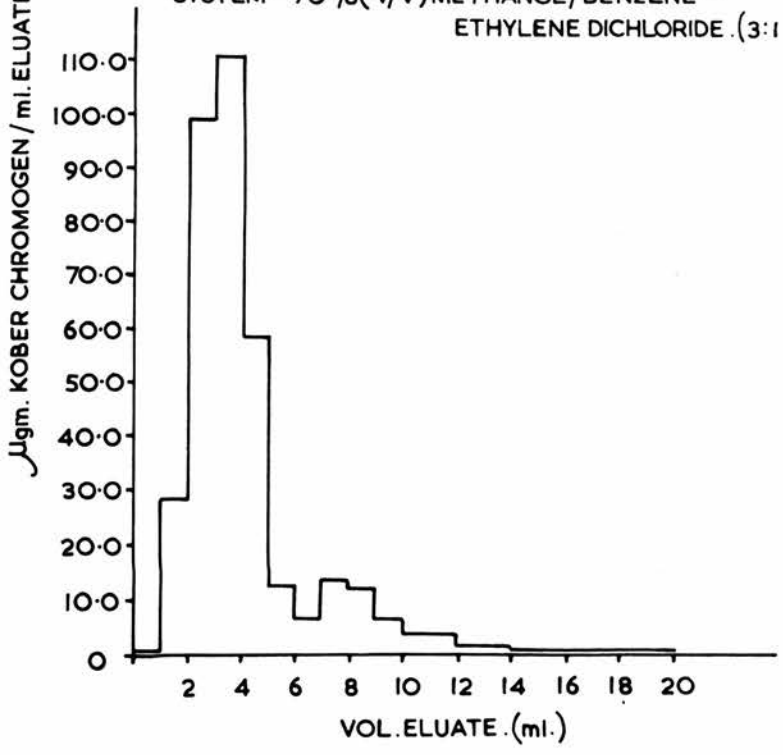
(h) Melting Point Determinations

The melting points of 16-oxooestradiol-17β, 16α-hydroxyoestrone (oestra-1:3:5-triene-3:16α-diol-17-one), KC-5, oestriol and 16-epi-oestriol were determined in sealed evacuated capillary tubes, while for those of the acetates of these substances, a microscope hot-stage was employed. The same thermometer was used in all determinations and the values given are uncorrected for emergent stem.

3. The Detection of a Fifth Kober Chromogen in an Extract of Pregnancy Urine.

A 24 hour sample of urine excreted by a woman in late pregnancy was hydrolysed by acid. The cooled solution was extracted four times with approximately one quarter of its volume of ether. The total ether extract was then washed once with one fifth of its volume of NaOH/$NaHCO_3$ buffer of pH 10.5. After shaking the ether extract with one twentieth of its volume of 2 N-NaOH, one fifth of its volume of 8.5% (w/v) $NaHCO_3$ solution was added, the mixture reshaken and the aqueous layer discarded. Finally, the ether/

FIG.9. CHROMATOGRAPHY OF PHENOLIC KETONES FROM HALF OF A 24 HRS SAMPLE OF PREGNANCY URINE.
SYSTEM – 70% (V/V) METHANOL / BENZENE – ETHYLENE DICHLORIDE. (3:1 BY VOL).



ether solution was washed once with one twentieth of its volume of 8.5% (w/v) $NaHCO_3$, three times with one fiftieth of its volume of water and evaporated to dryness.

The ketonic portion of this ether-soluble 'neutral + phenolic' fraction was separated by means of a Girard reaction, the residue being refluxed for one hour in 5 ml. of ethanol and 5 ml. of glacial acetic acid with 400 mg. of Girard's reagent T. The ketonic fraction was then obtained as described in Section III,2.

The ketonic portion was further fractionated by dissolving it in a few drops of ethanol and 50 ml. of benzene and extracting this solution twice with 25 ml. of N-NaOH. The alkaline phase was brought to pH 9.0-9.3 with CO_2 and extracted three times with 40 ml. of ether. After being washed with small volumes of 8.5% (w/v) $NaHCO_3$ and water, the ether extract was evaporated to dryness.

Half of this phenolic ketonic fraction was chromatographed on a micro column with the system 70% (v/v) methanol/benzene: ethylene dichloride (3:1 by vol.). TwoKober chromogens were eluted (Fig. 9). The first, eluted in the fraction/

fraction 1-6 ml., closely resembled oestrone in chromatographic behaviour, while the second, more 'polar' one was detected in the material eluted from 7-12 ml. In this partition system, the latter behaved exactly as does 16-oxooestradiol-17β. The amounts of each chromogen calculated to be present in the 24 hour urine sample were 619 µg. of oestrone and 81 µg. of the new chromogen, KC-5.

4. Preliminary Experiments on KC-5.

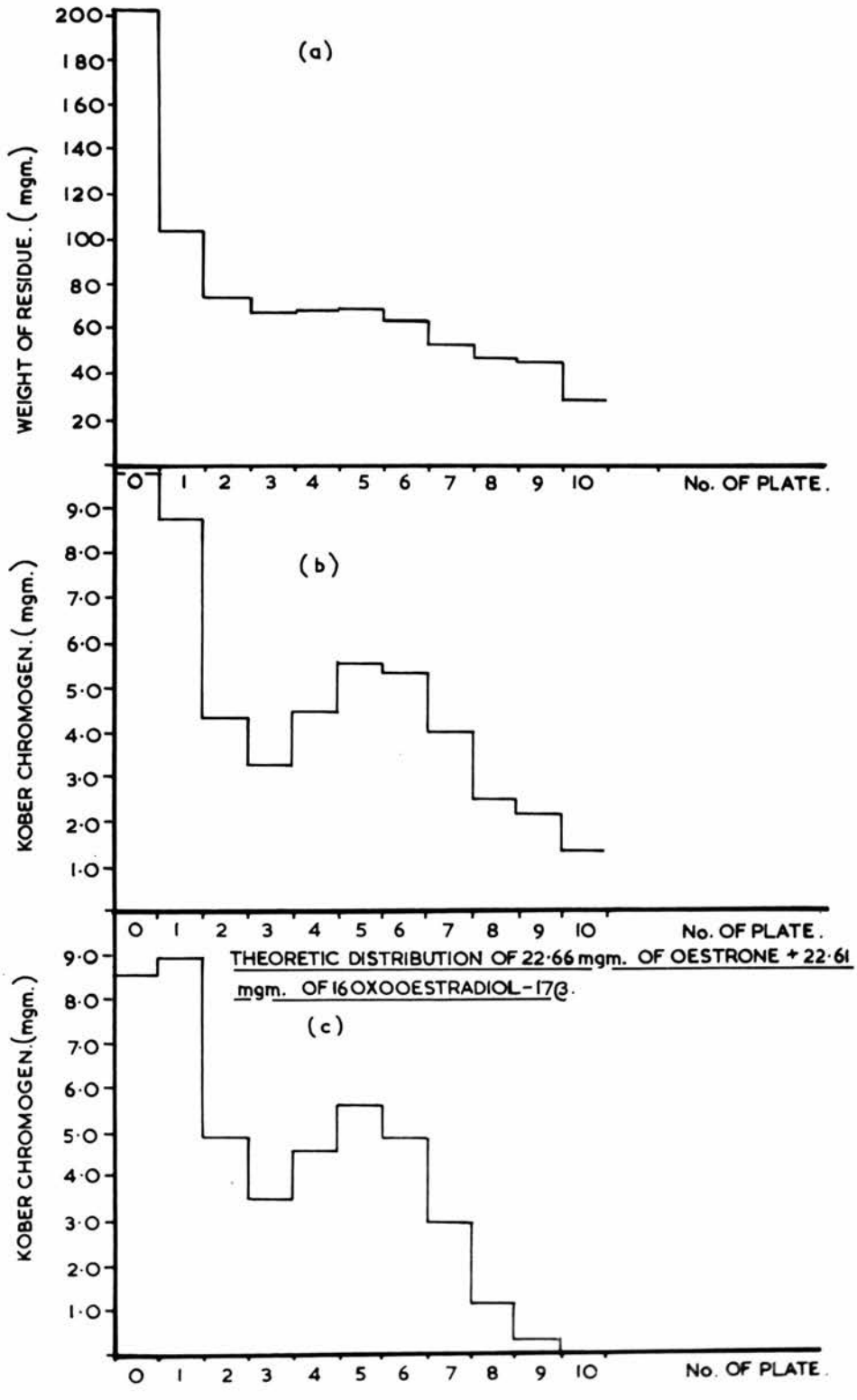
(a) Attempted isolation of KC-5 from acid-hydrolysed urine.

(i) Extraction of the phenolic ketonic fraction from urine.

Late pregnancy urine (200 litres) was acid-hydrolysed in batches of 4 litres. After 750 g. of NaCl had been added to each batch, the solution was cooled and extracted once with an equal volume of ether. The ether extract was washed once with 400 ml. of 5% (w/v) $NaHCO_3$ and once with 400 ml. of H_2O. The phenolic fraction was then separated by the usual alkaline extraction (Section III,2).

The/

FIG.10. COUNTER CURRENT DISTRIBUTION OF THE PHENOLIC KETONIC FRACTION FROM 200 LITRES OF ACID HYDROLYSED PREGNANCY URINE.



The phenolic fractions from each 50 litres of urine were subjected to a Girard separation which employed 15 minutes' heating under reflux in the initial reaction.

The combined ketonic phenolic fraction so obtained from 200 litres of urine weighed 828 mg. This extract was obviously grossly impure.

(ii) Purification of the phenolic ketonic fraction by countercurrent distribution.

The solvent system chosen was benzene-n-hexane (4:1 by vol.)/50% (v/v) ethanol. A ten transfer distribution was carried out in separating funnels, using 50 ml. of each phase. The mobile phase was the lower aqueous alcoholic one. The average temperature during the distribution was 18.0°C.

The contents of each funnel were evaporated to dryness *in vacuo*, the residue weighed (Fig. 10a) and the Kober chromogen content of each plate estimated (Fig. 10b). From the latter graph, the amounts of oestrone and KC-5 estimated to be present in the total phenolic ketonic fraction were 22.66 mg. and 22.61 mg. respectively. The partition/

partition coefficients of oestrone and of 16-oxo-oestradiol-17β in the system used were determined as 9.8 and 0.95 respectively. The theoretical distribution of a mixture of 22.66 mg. of oestrone and 22.61 mg. of 16-oxooestradiol-17β was hence calculated (Fig. 10c). A comparison of this theoretical distribution with the one experimentally determined indicated a marked similarity between the behaviour of the urinary KC-5 and that of 16-oxooestradiol-17β.

(iii) Partition chromatography of the KC-5 fraction

The contents of plates 3-7 were combined and chromatographed on a column 4 cm. in diameter with the system 70% (v/v) methanol/benzene: ethylene dichloride (3:1 by vol.). The column was prepared with 80 g. of Celite, in the usual type of slurry. The fraction to be chromatographed was dissolved in 10 drops of methanol and 15 ml. of mobile phase. A further 15 ml. of mobile phase was used to effect its quantitative transfer on to the top of the column. Twelve 30 ml. portions of eluate were collected and analysed for Kober chromogens. The more 'polar' KC-5 was eluted in fractions

8 /

8-12. These fractions, representing 14 mg. of KC-5, were combined and a number of unsuccessful attempts were made to remove further impurities by treating the fraction with various solvents. It was finally decided that the degree of contamination was too high to achieve isolation of KC-5 in crystalline form.

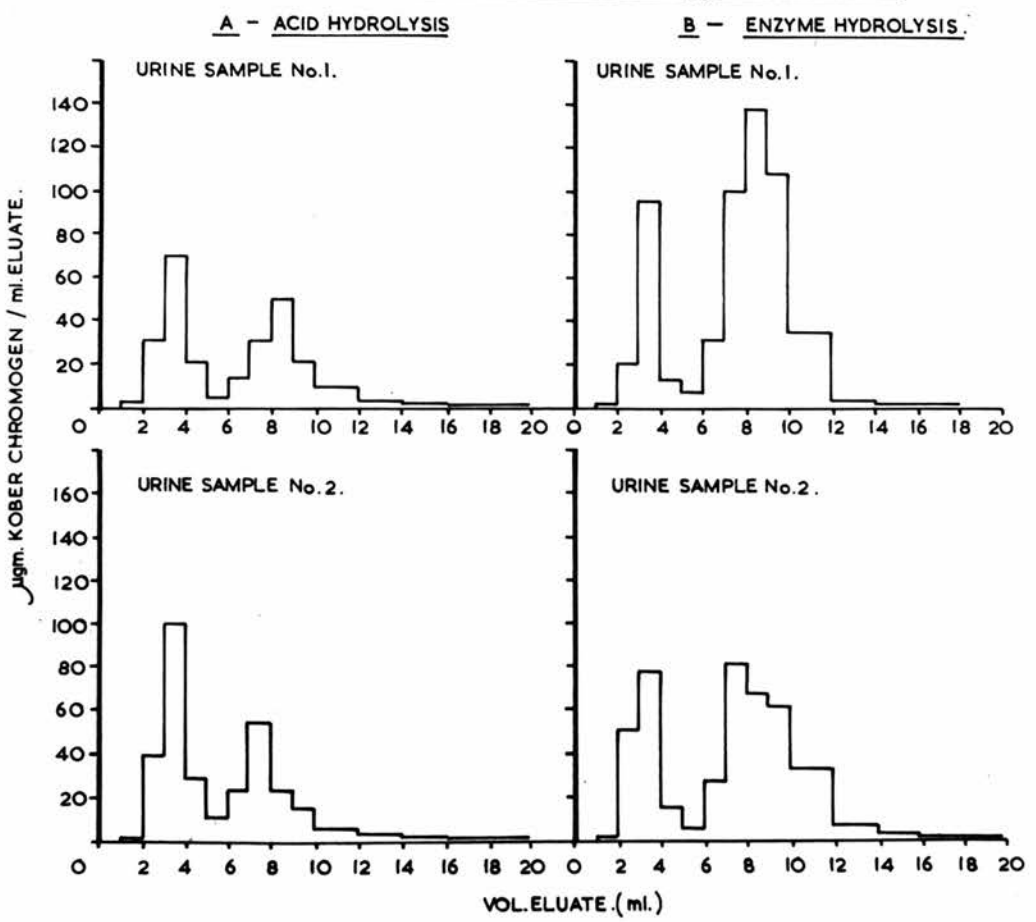
All the evidence up to this point, however, indicated a close resemblance between KC-5 and 16-oxooestradiol-17β; i.e. their behaviour in partition chromatography in two different solvent systems was identical and their distribution between the two phases of yet a third solvent system was very similar.

(b) <u>A comparison of the yields of KC-5 from pregnancy urine after hot acid hydrolysis with those obtained after hydrolysis with β-glucuronidase</u>.

In order to find out if KC-5 was being produced from another oestrogen during acid hydrolysis of the urine, a comparison was made of the yields of KC-5 from pregnancy urine after hot acid hydrolysis with those obtained after incubation with β-glucuronidase.

Each/

FIG. II. A COMPARISON OF THE YIELDS OF KC-5 FROM PREGNANCY URINE AFTER HOT ACID HYDROLYSIS WITH THOSE OBTAINED AFTER HYDROLYSIS WITH β-GLUCURONIDASE.



Each 24 hour sample of pregnancy urine was divided into two equal portions, the first of which was hydrolysed by hot acid, the second by water-soluble β-glucuronidase from Patella vulgata. The phenolic ketonic fraction of each hydrolysed portion was separated by a method identical with that described for the original detection of KC-5 (Section III,3), and chromatographed on a micro column with the system 70% (v/v) methanol/ benzene:ethylene dichloride (3:1 by vol.)(Fig.11). The yields of oestrone and of KC-5 are shown in Table 2.

Table 2. A comparison of the yields of KC-5 from pregnancy urine after hot acid hydrolysis with those obtained after hydrolysis with β-glucuronidase.

Urine	Method of hydrolysis	Oestrone/24 hr. sample (μg.)	KC-5/24 hr. sample (μg.)
1	Acid	286	307
1	Enzyme	294	1022
2	Acid	385	307
2	Enzyme	326	719

It was immediately evident that, whereas the yield of oestrone did not appreciably vary with the method of hydrolysis, the amount of KC-5 detected/

detected in the final fraction from enzyme-hydrolysed urine was two or three times greater than the yield from acid-hydrolysed urine. It was concluded that hydrolysis of urine with hot acid certainly does not produce KC-5 and in fact it destroys a large percentage of the KC-5 present.

(c) Recovery experiments with 16-oxooestradiol-17β

In preliminary experiments with 16-oxooestradiol-17β, virtually no destruction was observed after one hour's boiling in water with 15 vols.% of 10 N-HCl. However, when a sample of 16-oxooestradiol-17β was boiled in male urine under similar conditions and taken through the entire fractionation procedure, only 4% was recovered. A 'control' extraction carried out on 16-oxooestradiol-17β which had been boiled in water with HCl for one hour, rather surprisingly gave a recovery of only 22%, indicating considerable destruction during some part of the fractionation as well as destruction during the preliminary boiling with acid.

The/

The Girard separation was at once suspected since it involved heating the urine extracts in an acid solution.

100 μg. portions of 16-oxooestradiol-17β were therefore subjected to a Girard separation under varying conditions. Each sample was treated with 2 ml. of ethanol, 2 ml. of glacial acetic acid and 100 mg. of Girard's reagent T. The ketonic fraction was obtained in the usual way and its Kober chromogen content measured. The results (Table 3) indicate that heating the initial reaction mixture for even a short time, causes considerable destruction of 16-oxooestradiol-17β.

Table 3. Recovery of 16-oxooestradiol-17β from the Girard reaction carried out under varying conditions.

Conditions for initial reaction	Recovery %
17 hours at room temperature	95.2
5 minutes' heating under reflux	66.0
15 minutes' heating under reflux	64.8
60 minutes' heating under reflux	41.6

In all subsequent work, therefore, the Girard reaction was allowed to proceed overnight at room temperature.

Other/

Other modifications in the method used to detect KC-5 in urine were suggested by recovery experiments with 16-oxooestradiol-17β carried out by my colleague Mr. K.H.Loke (personal communication). He found that the separation of the phenolic fraction was effected more readily by alkali extraction from ether than from benzene solution; alkali extraction had to be carried out with chilled solutions and with the minimum of delay so that the destruction of 16-oxo-oestradiol-17β in alkali was reduced; ether had to be free from peroxides. Since it was also observed that peroxide formation was decreased by storing the ether dry, all ether solutions were dried over Na_2SO_4 before evaporation of the ether.

All these modifications were incorporated into the method used to isolate KC-5 from enzyme-hydrolysed pregnancy urine and, later, in the method used to detect KC-5 in menstrual cycle urines.

5. Isolation of KC-5 from enzyme-hydrolysed pregnancy urine.

Urine excreted by women in late pregnancy was hydrolysed by whole enzyme and the phenolic ketonic fractions/

fractions separated by means of the methods described in Section II,2 and Section III,2. Chromatographic analysis on a micro column of aliquots of such fractions obtained from each batch of approximately 100 litres of urine showed a relatively constant content of KC-5 and of oestrone (Table 4).

Table 4. Ketonic-phenolic fractions from enzyme-hydrolysed late-pregnancy urine: Oestrone and KC-5 contents.

Batch	Vol.of urine (l.)	Wt. of ketonic phenolic fraction mg.	Oestrone content (%)	KC-5 content (%)
I	75	207	3.5	15
II	101	377	3.5	14
III	100	284	3.4	22
IV	95	274	4.4	15

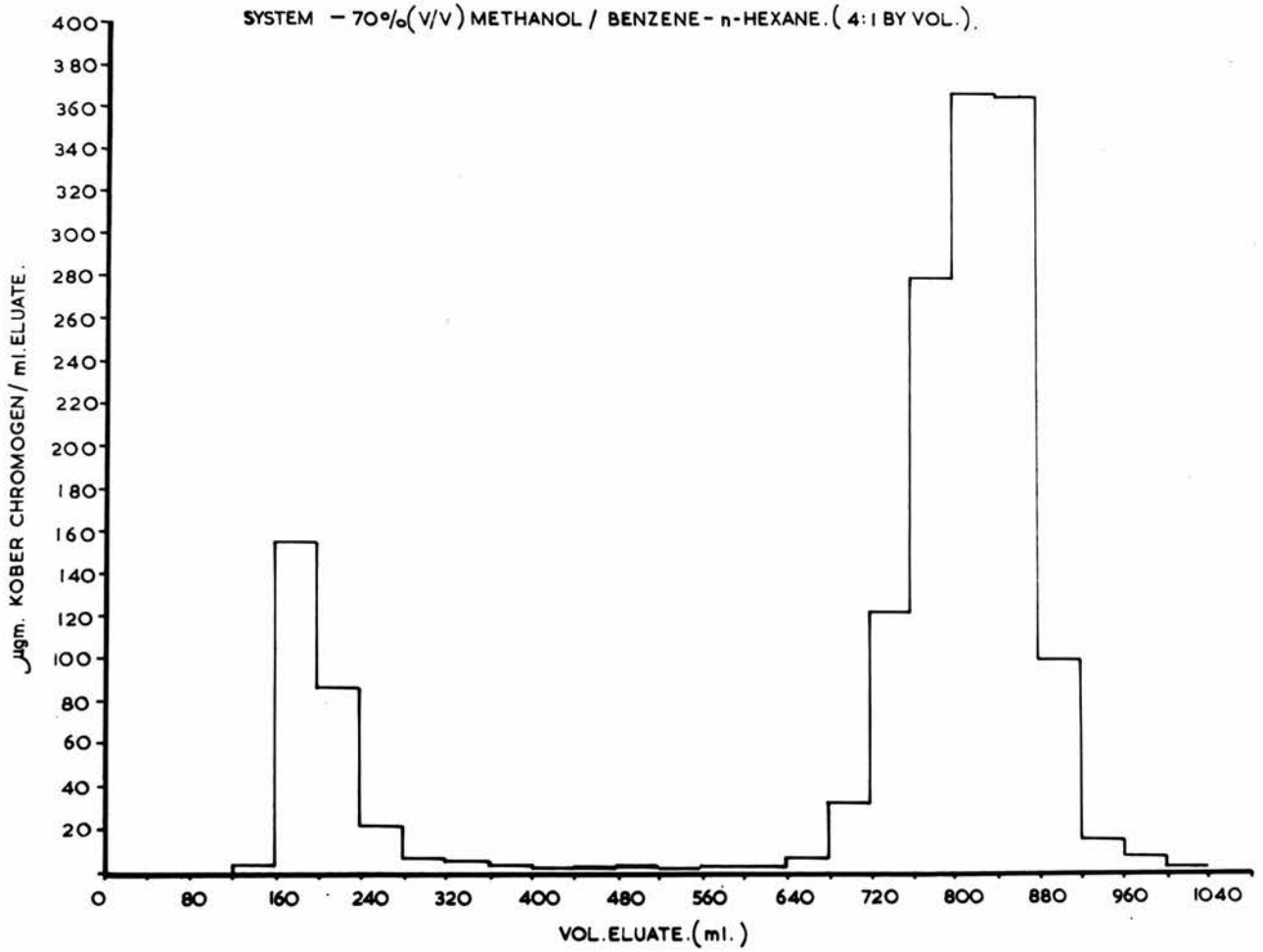
The ketonic phenolic fraction from approximately 100 litres of urine (Batch II) was dissolved in 75 ml. of each of 50% (v/v) ethanol and benzene-n-hexane (4:1 by vol.) and distributed between these phases. The lower layer was separated and the upper layer re-extracted twice with 75 ml. of lower phase. The combined lower phases were chromatographed on a 4 cm. diameter column/

column with the system 70% (v/v) methanol/benzene-n-hexane (4:1 by vol.). The KC-5 fractions were combined and acetylated. The acetate was re-crystallized several times from a mixture of ethyl acetate and n-hexane. This acetate melted at 172°C. (cf. 16-oxooestradiol-17β diacetate, m.p. 132.5-134°C.). It gave the typical pink colour in the Kober reaction. Chromatography with the system 90% (v/v) ethanol/n-hexane showed that the unknown acetate behaved identically with the diacetate of 16-oxooestradiol-17β. The optical rotation of an ethanolic solution of the unknown acetate gave $[\alpha]_D^{12.5} = +159$ (cf. 16-oxooestradiol-17β diacetate $[\alpha]_D = -69.5$ (in ethanol)). The strong dextro rotation suggested that if the ketonic group was in ring D, its position was more likely to be C_{17} than C_{16} (Barton and Klyne, 1948). Unfortunately, there was insufficient of the acetate to complete the identification. It was quite certain, however, that contrary to expectation, KC-5 was not identical with 16-oxo-oestradiol-17β.

Two further batches of pregnancy urine (III and IV), each consisting of approximately 100 litres/

FIG. 12. PARTITION CHROMATOGRAM OF PHENOLIC KETONES FROM BATCH III ENZYME-HYDROLYSED PREGNANCY URINE.

SYSTEM – 70% (V/V) METHANOL / BENZENE – n-HEXANE. (4:1 BY VOL.).



litres, were hydrolysed by enzyme and the phenolic ketonic fraction chromatographed with the system 70% (v/v) methanol/benzene-n-hexane (4:1 by vol.) on a column 2 cm. in diameter as described in Section III,2. The elution pattern of the first of these chromatograms is shown in Fig. 12. The combined KC-5 fractions from this chromatogram weighing 55.3 mg., were estimated to contain 45.5 mg. of Kober chromogen. After purifying this fraction further by treating it with small volumes of chilled methanol, 35.0 mg. of an almost white solid remained.

Chromatography of the phenolic ketones from batch IV of pregnancy urine yielded a concentrate of KC-5 weighing 47 mg. and containing 23.8 mg. of Kober chromogen. Treatment of this residue with chilled methanol gave 15.7 mg. of solid KC-5.

These two final KC-5 fractions were combined and crystallized from methanol to yield 9.2 mg. of 'Grade I' KC-5 (m.p. 236.5-238.5°C.). The mother liquor and methanolic washings were combined, evaporated to dryness and retreated with small/

small volumes of chilled methanol. In this way, two further batches of crystalline KC-5 were obtained - 24.6 mg. of 'Grade II' material of m.p. 235-238°C. and 15.3 mg. of 'Grade III' material, m.p. 234-238°C.

6. Preliminary Identification of KC-5.

(a) Melting point determinations.

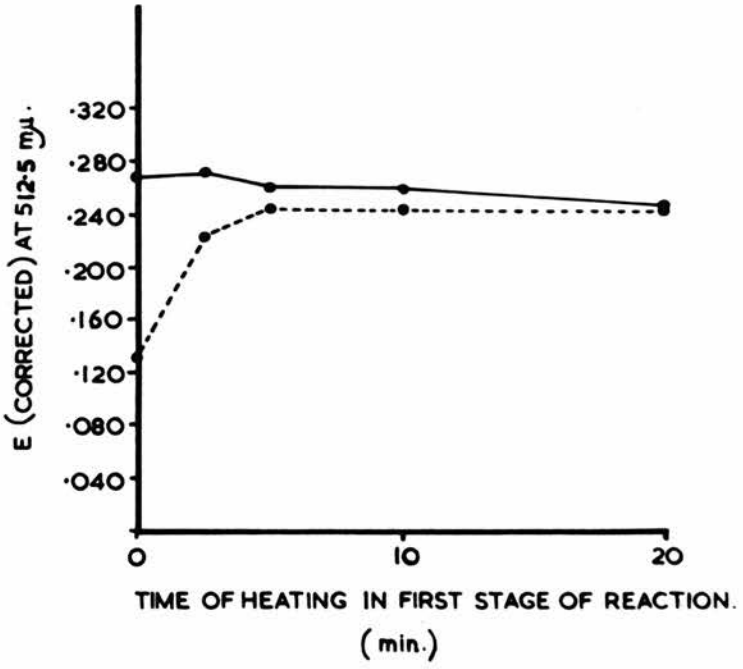
The melting point of recrystallized KC-5 (Grade I) after drying for 2 hours at 100° in vacuo was 236.5-238.5°C. with preliminary shrinkage and sublimation from about 210°C. onwards. A mixed melting point with 16-oxo-oestradiol-17β (238-241°C.) was determined as 235-240.5°C. with, again, preliminary shrinkage from 210°C. onwards. There was, therefore, no significant depression of the melting point of KC-5 on admixture with 16-oxooestradiol-17β.

(b) Analysis.

Analysis of the recrystallized KC-5 gave C, 75.50%; H, 7.91%. This was in excellent agreement with the theoretical values for a substance of the formula $C_{18}H_{22}O_3$, viz. C, 75.49%; H, 7.75%.

(c) /

FIG.13. EFFECT OF VARIATION IN TIME OF HEATING IN THE FIRST STAGE OF THE KOBER REACTION ON INTENSITIES OF FINAL COLOURS DEVELOPED BY 16 OXO-OESTRADIOL-17β (•------•) AND KC-5 (•——•).



(c) Optical rotation.

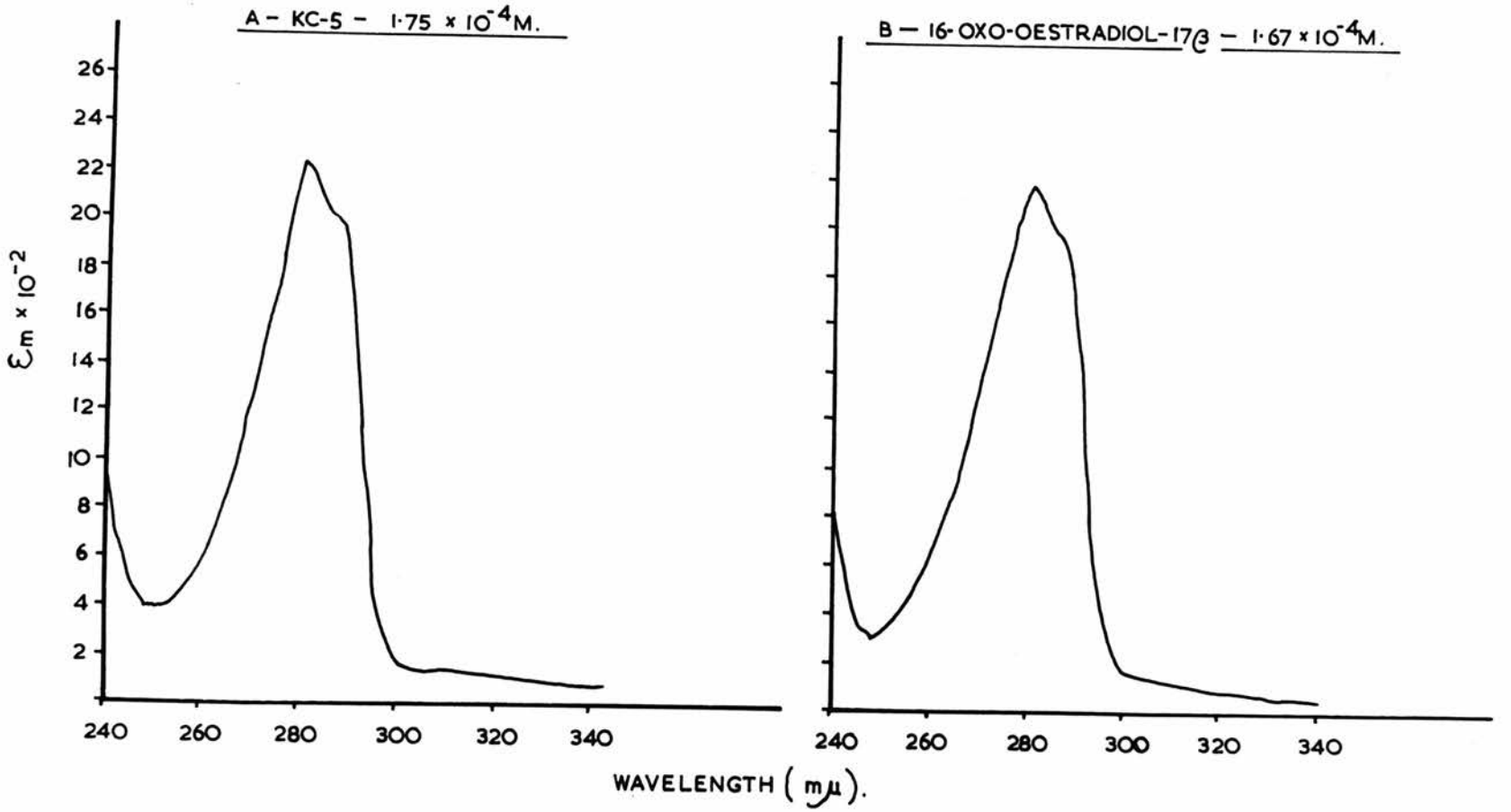
Although the melting point and the results of analysis of KC-5 agreed excellently with the expected values for 16-oxooestradiol-17β, the optical rotation measurements clearly indicated that KC-5 could not be identical with 16-oxo-oestradiol-17β. Grade I KC-5 had $[\alpha]_D^{15}$ = +143° (in ethanol) while 16-oxooestradiol-17β gave $[\alpha]_D^{12}$ = -89° (in ethanol).

(d) Kober colour.

Aliquots of an ethanolic solution of re-crystallized KC-5 were assayed by the Kober reaction using varying times of heating in the first stage of the reaction. All the tubes were given the usual 15 minutes' heating during the second stage of the reaction. The results are shown in Fig. 13. Since KC-5 developed its maximum intensity of colour with no heating in the first stage of the reaction whereas 16-oxo-oestradiol-17β requires 5 minutes' heating, this was a second indication that KC-5 differed from 16-oxo-oestradiol-17β.

(e) /

FIG. 14. ULTRAVIOLET ABSORPTION SPECTRA OF KC-5 AND 16-OXO-OESTRADIOL-17β IN ETHANOLIC SOLUTION.



(e) Absorption spectra of ethanolic solutions of KC-5 and of 16-oxooestradiol-17β.

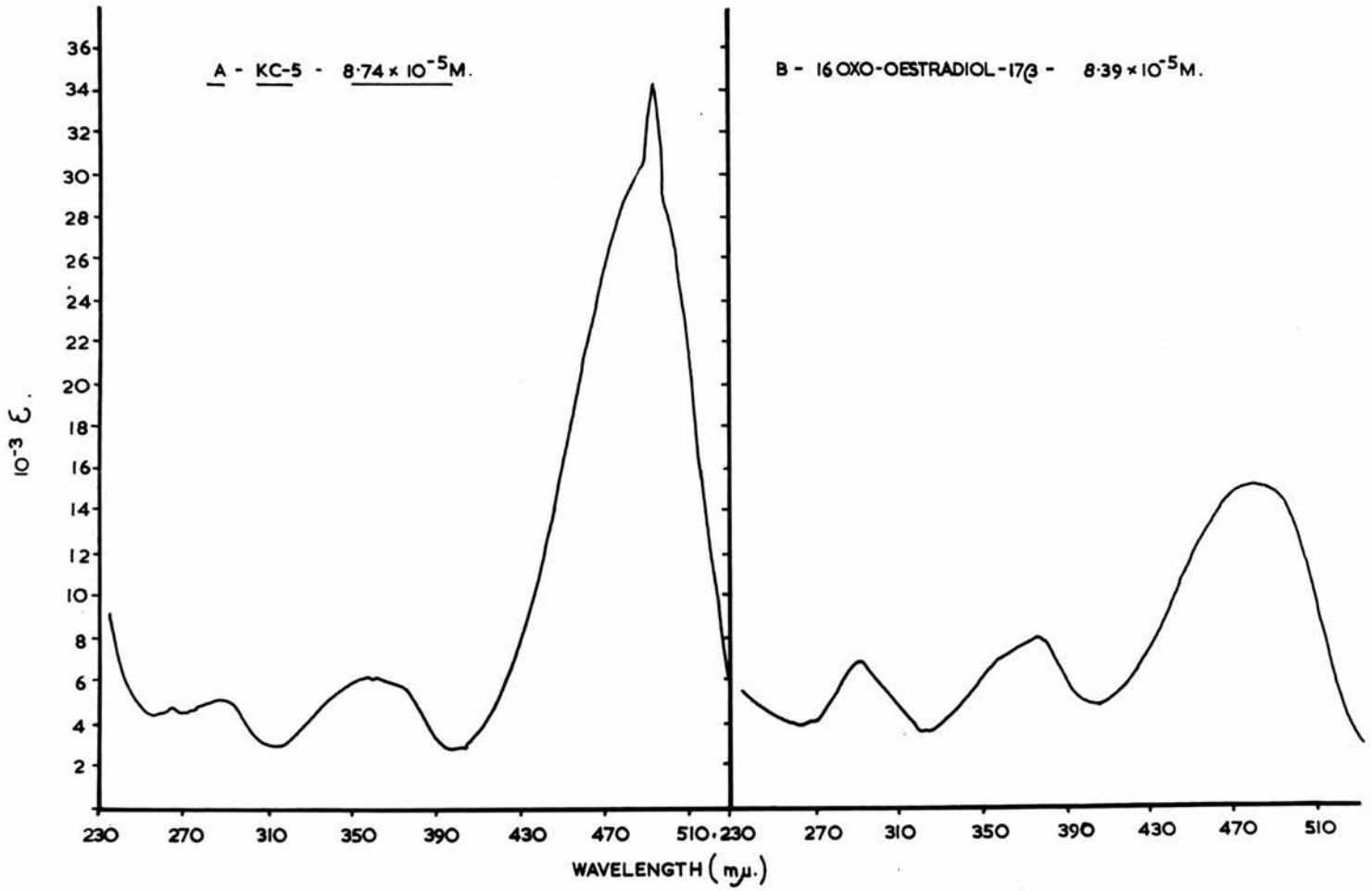
The absorption spectra of solutions of Grade I KC-5 and of 16-oxooestradiol-17β in ethanol were measured over the range 220-400 mμ. As is apparent from Fig. 14, these spectra were virtually identical over this range.

(f) Sulphuric acid spectra of KC-5 and 16-oxo-oestradiol-17β.

Another very striking difference between KC-5 and 16-oxooestradiol-17β was observed when about 100 μg. of each were treated with 4 ml. of concentrated H_2SO_4. With H_2SO_4, KC-5 gave an immediate red colour. Within a few minutes, green fluorescence became visible. After two hours' incubation at 24°C., the absorption spectrum of the solution was measured over the range 215-600 mμ.

When 16-oxooestradiol-17β was similarly treated, no immediate red colour developed. A green fluorescence became visible during the first few minutes and, although, during the incubation at 24°C., some red colour developed, its intensity was/

FIG. 15. ABSORPTION SPECTRA OF H_2SO_4 SOLUTIONS OF KC-5 & 16 OXO-OESTRADIOL-17β AFTER 2HRS AT 24°C.



was still considerably less than that developed with KC-5. The absorption spectra are shown in Fig. 15.

(g) Blue tetrazolium reaction.

The method used for this reaction was that described by Mader and Buck (1952). To 15μg. portions of KC-5 and 16-oxooestradiol-17β respectively, were added 0.2 ml. of 95% (v/v) ethanol, 0.04 ml. of 5% (v/v) tetramethyl ammonium hydroxide in 95% (v/v) ethanol and 0.3 ml. of a solution of blue tetrazolium (3,3'-dianisole-bis-4,4'-(3,5-diphenyl) tetrazolium chloride) containing 0.100 g. of the tetrazolium salt in 12 ml. of 95% (v/v) ethanol. The mixtures were stoppered and incubated at 24°C. for 1 hour after which 0.04 ml. of 10% (v/v) acetic acid in 50% (v/v) ethanol and 10 ml. of 95% (v/v) ethanol were added. The optical densities of the solutions were measured at 510 mμ. The intensity of colour developed by KC-5 was found to be only one third of that developed by 16-oxooestradiol-17β, indicating that KC-5 is not such a strong reducing agent as 16-oxooestradiol-17β.

(h)/

(h) Partition chromatography.

Grade I KC-5 and 16-oxooestradiol-17β were each chromatographed on a micro column with the system 70% (v/v) methanol/benzene-n-hexane (4:1 by vol.). Each of these substances was eluted in the fraction 12-20 ml.

(i) Zimmerman reaction.

50 µg. of KC-5 were treated with 0.2 ml. of ethanol, 0.2 ml. of ethanolic KOH (2.5N in absolute ethanol) and 0.2 ml. of a 2% (w/v) solution cf m-dinitrobenzene in ethanol. After this mixture had been incubated for one hour at 25°C. in the dark, 3 ml. of ethanol were added. No purple colour developed. It can therefore be concluded that if KC-5 has its ketonic group in ring D and is a derivative of oestrone, ~~and~~ as suggested by its optical rotation (Barton and Klyne, 1948), it must also have a substituent group on C16 (Callow, Callow and Emmens, 1938).

(j) Acetylation of KC-5.

15.1 mg. of KC-5 were treated overnight at room temperature with 0.5 ml. of anhydrous pyridine and 0.5 ml. of acetic anhydride. Crushed/

Crushed ice was then added until the volume was 10 ml. After one hour, the precipitate was separated, thoroughly washed and dried. The product, weighing 11.4 mg., was crystallized from a mixture of n-hexane and ethyl acetate and had a melting point of 166-167°C. with droplets appearing from 160°C. onwards. The mixed melting point with the diacetate of 16-oxooestradiol-17β (132.5-134°C.) was 120-142°C.

Analysis of the product showed C, 71.22%; H, 6.84%. (Theoretical values for $C_{22}H_{26}O_5$ are C, 71.33%; H, 7.08%).

The optical rotation gave $[\alpha]_D^{13}$ = +156° (in ethanol).

(k) Reduction of KC-5 by sodium borohydride.

The ketonic group of both oestrone and 16-oxooestradiol-17β is reduced by $NaBH_4$ to produce a β-OH group, i.e. oestrone was found by Biel (1951) to give rise exclusively to oestradiol-17β and Huffman and Lott (1955) achieved almost quantitative reduction of 16-oxo-oestradiol-17β to 16-epioestriol. Both of these observations were verified under the conditions which were to be used for the reduction/

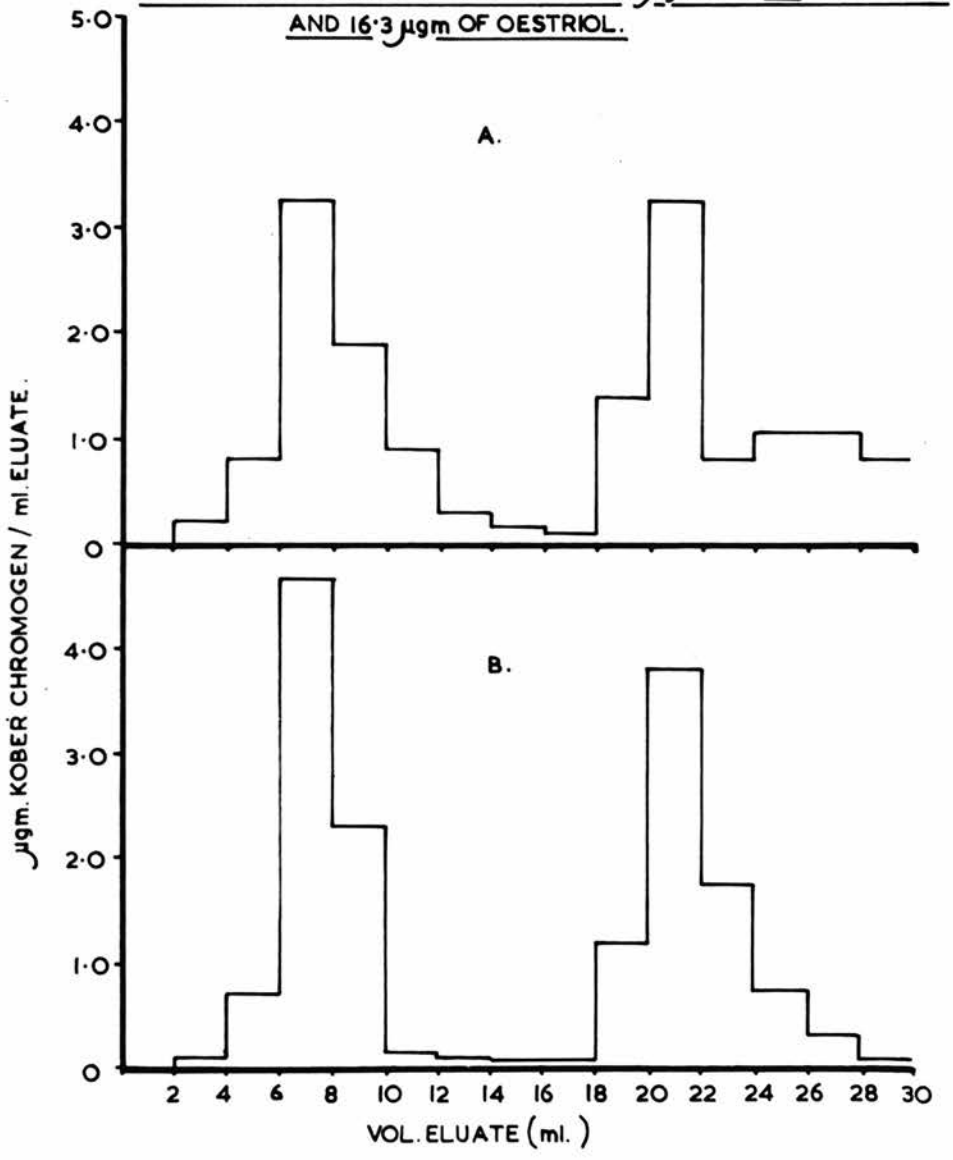
reduction of KC-5. It was therefore expected that if KC-5 were a pure substance and did not suffer any rearrangement during the reaction it would give rise to only one reduction product.

The reduction was effected by dissolving 20 mg. of Grade II KC-5 in 10 ml. of methanol. $NaBH_4$ (15 mg.) was added and the solution left at room temperature for 40 minutes. After the addition of a further 10 mg. of $NaBH_4$, the reaction was allowed to continue for one hour. About half of the methanol was evaporated off under reduced pressure in a warm water bath and, after dilution with 100 ml. of water and acidification with 4 ml. of 10 N-HCl, the mixture was extracted thoroughly with ether. The ether extract was washed with aqueous $NaHCO_3$ and with water and evaporated to dryness.

The product was treated at room temperature for 17 hours with 250 mg. of Girard's reagent T in 5 ml. of ethanol and 5 ml. of glacial acetic acid. The non-ketonic material obtained in the usual way was found to weigh 20.2 mg.

Chromatography of one five-hundredth of this product on a micro column with the system 70% (v/v) methanol/ethylene dichloride indicated the/

FIG.16. ANALYTICAL PARTITION CHROMATOGRAMS OF A – $NaBH_4$ REDUCTION PRODUCT OF KC-5 & B – A MIXTURE OF 15·5 µgm OF 16 EPIOESTRIOL AND 16·3 µgm OF OESTRIOL.



the presence of two Kober chromogens - 7.9 mg. of the less 'polar' component which was eluted in the fraction 4-12 ml. and 9.6 mg. of the more 'polar' chromogen eluted in the fraction 18-22 ml. For comparison, a mixture of 15.5 µg. of 16-epioestriol and 16.3 µg. of oestriol was chromatographed in the same system (Fig. 16).

The main bulk of the product was separated by chromatography on a 2 cm. diameter column with the same system. The less and more 'polar' fractions obtained weighed 6.4 and 7.8 mg., respectively.

After one crystallization from methanol-benzene, the less 'polar' product melted at 273-276°C. and the mixed melting point with 16-epioestriol (m.p. 284-285°) was 274-281°C. The remainder of the crystalline material, together with that obtained by evaporation of the mother liquor, was acetylated in the usual way with acetic anhydride and anhydrous pyridine at room temperature. The product, after one crystallization from n-hexane, melted at 151-152°C. and the mixed melting point with 16-epioestriol triacetate (m.p. 151-155°) was 150-152°C. This was considered satisfactory evidence for the presence/

presence of 16-epioestriol in the less 'polar' fraction.

The more 'polar' fraction of the reduction product gave a substance melting at 277-279°C. after one crystallization from methanol-benzene, and the mixed melting point with oestriol (m.p. 279-280°C.) was 277-280°C. Oestriol, therefore, was present in the more 'polar' fraction.

Contrary to expectations, therefore, KC-5 had yielded at least two products on reduction with $NaBH_4$ - 16-epioestriol and oestriol.

(1) Conclusions.

KC-5 on acetylation yielded a diacetate melting at 166-167°C. in marked contrast to the diacetate of 16-oxooestradiol-17β which melts at 132.5-134°C. This observation suggested that KC-5 might be 6-oxooestradiol-17β, the diacetate of which melts at 173-175°C. (Longwell and Wintersteiner, 1940). To test this possibility, the ultraviolet absorption of a solution of KC-5 in ethanol was measured. The spectrum closely resembled those of 16-oxooestradiol-17β, oestriol, 16-epioestriol and oestrone and did not show the enhanced absorption characteristic of the extended/

extended conjugation at C6 in 6-oxooestradiol-17β, as recorded by the above authors.

In the blue tetrazolium test, as modified by Mader and Buck (1952) KC-5 showed considerable reducing power indicating that it might contain an α-ketol grouping. However, the intensity of the colour produced in this test was only about one third of the colour produced by an equivalent amount of 16-oxooestradiol-17β. This observation recalled a similar finding by Fotherby and Marrian (1957) where 16α-hydroxyandrost-4-ene-3:17-dione (Fried, Thoma, Perlman, Herz and Borman, 1955) was found to give a considerably less intense colour in this reaction than those given by either 16-oxooestradiol-17β or preg-4-ene-17α:21-diol-3:20-dione. It seemed possible therefore that KC-5 might be a 16-hydroxy derivative of oestrone.

This idea was supported by the evidence of the strong dextro rotation of KC-5 and of its diacetate which is consistent with the existence in the molecule of a C=O on C17 (cf. Barton and Klyne, 1948). Also, if KC-5 were a derivative of oestrone the negative result in the Zimmerman reaction would indicate a substituent group on C16/

C16.

It was expected that $NaBH_4$ reduction of KC-5 would yield only one product, so establishing the configuration of the probable OH group on C16. Unfortunately, due to shortage of available material, the reduction had to be carried out on a sample of KC-5 which had been recovered from the mother liquors of the analytical sample.

Assuming that $NaBH_4$ reduction cannot cause inversion of either a C-16 or a C-17 hydroxyl group and that reduction of the ketonic group in ring D gives rise to a β-OH group only, then the KC-5 used in the reduction must have contained 16α-hydroxyoestrone in order to account for the presence of oestriol in the reduction products. 16-Epioestriol, however, could be produced by $NaBH_4$ reduction of 16β-hydroxyoestrone, of 16-oxooestradiol-17β or of both. It seems probable, then, that the sample of KC-5 used in the reduction contained 16α-hydroxyoestrone and, in addition, one or both of 16β-hydroxyoestrone and 16-oxooestradiol-17β.

The recrystallized KC-5, itself, might not have been a pure substance, but it seems certain/

certain that the major component was either 16α or 16β-hydroxyoestrone and not the strongly laevorotatory 16-oxooestradiol-17β. If 16α-hydroxyoestrone was the principal component of the recrystallized material, then the impurities which, of course, would become more concentrated in the mother liquors, might be 16β-hydroxyoestrone and/or 16-oxo-oestradiol-17β. If, on the other hand, 16β-hydroxyoestrone was the principal component, then 16α-hydroxyoestrone must have been a contaminant, with or without 16-oxo-oestradiol-17β.

There is another possible source of 16-oxooestradiol-17β in the KC-5 extracts apart from its actual occurrence in pregnancy urine. Cooley, Ellis, Hartley and Petrov (1955) have shown that alkaline hydrolysis at room temperature of androst-5-ene-3β:16α-diol-17-one diacetate yields androst-5-ene-3β:17β-diol-16-one. It is therefore not improbable that if 16α-hydroxy-oestrone was present in the original urinary extracts, the analogous rearrangement might have occurred during the extraction with aqueous alkali of the phenolic fraction of urine/

urine.

Finally, there is the possibility that $NaBH_4$ reduction of, say, 16α- or 16β-hydroxy-oestrone might give rise to more than one product. Also, although 16-epioestriol and oestriol were definitely shown to be present in the less and more 'polar' fractions respectively, of the reduction product, there may have been small amounts of other Kober chromogens present along with each of these triols. For instance, in the chromatographic system used, 16-epioestriol and 17-epioestriol (oestra-1:3:5-triene-3:16α:17α-triol)(Prelog, Ruzicka and Wieland,1945) can scarcely be differentiated. Small amounts of 17-epioestriol in the less 'polar' fraction might therefore have escaped detection.

From a superficial study of the molecular model of ring D of the fourth possible triol, oestra-1:3:5-triene-3:16β:17α-triol, it seems likely that it would be slightly more 'polar' than oestriol and might possibly be eluted just behind oestriol in the chromatographic system used. Consequently, if such an observation

is/

is justified, the 'oestriol' fraction of the reduction product might have contained small amounts of this triol. The chromatogram of an aliquot of the reduction product (Fig.16) showed less sharp elution peaks than did the mixture of authentic oestriol and 16-epioestriol. This could be due to the presence of more than one Kober chromogen in each fraction but, of course, might be caused by some quite unrelated contaminants.

It was decided that more KC-5 would have to be isolated from urine and rigorously purified, in order to establish its identity with absolute certainty.

7. The Stability of KC-5.

(a) Stability to boiling in acid with and without urine.

Two 150 µg. samples of recrystallized KC-5 were dissolved in 250 ml. of male urine and water, respectively. Each of these solutions was heated to boiling, then boiled for one hour under reflux with 15 vols.% of 10 N-HCl. Each solution was then taken through the usual extraction procedure. The Girard reaction was carried out at room temperature overnight. The

final/

final ketonic phenolic fractions were chromatographed in the system 70%(v/v) methanol/benzene-n-hexane (4:1 by vol.). 67% of the KC-5 was recovered from the sample which was boiled with acid and water and only 33% of the sample boiled with acid and urine.

This is further evidence that KC-5 is destroyed by acid hydrolysis in urine.

(b) Stability to the Girard reaction.

Two 50 µg. portions of recrystallized KC-5 were dissolved in 2 ml. of ethanol and 2 ml. of glacial acetic acid and treated with 100 mg. of Girard's reagent T. One such solution was left to react at room temperature overnight; the other was heated under reflux for 60 minutes. The ketonic fraction was then separated from each and the Kober chromogen content of each measured.

The recovery of KC-5 from the sample which had been left to react overnight was 85%, but from the sample which had been heated under reflux for one hour, only 58% was recovered.

The precaution of carrying out the Girard reaction at room temperature overnight is therefore fully justified.

8. /

8. Final Identification of KC-5 as 16α-hydroxyoestrone.

(a) Synthesis of 16α-hydroxyoestrone.

16α-Hydroxyoestrone diacetate was prepared from oestrone by my colleague, Mr K.H.Loke, (Marrian, Loke, Watson and Panattoni, 1957) using the method of Leeds, Fukushima and Gallagher (1954). After repeated recrystallization, this was found to have $[\alpha]_D^{18}$ +153° (ethanol) and +126° (chloroform) and a melting point of 172-174.5°C. The above mentioned authors recorded $[\alpha]_D^{28}$ +122° (chloroform) and melting point 179-180°C. for their preparation. However, it would appear that the latter higher melting point is that of a different polymorphic modification of the same substance, since a sample of the preparation of Leeds et al. (1954), kindly supplied to us by Dr Gallagher, was found to melt at 168.5-172°C. and this melting point was not depressed after admixture with Mr Loke's preparation.

Hydrolysis of the diacetate was effected with methanolic H_2SO_4 at room temperature somewhat as described by Leeds et al.(1954) for the rearrangement and hydrolysis of 16α:17α-epoxy-androstane-3β:17β-diol/

diol diacetate to 17-oxo-androstane-3β:16α-diol. After recrystallization from methanol, the product melted at 238-240.5°C. with marked shrinkage at about 216° and had $[\alpha]_D^{16}$ +180° (ethanol).

16α-Hydroxyoestrone was also prepared more conveniently by treatment of 16α:17α-epoxy-oestra-1:3:5-triene-3:17β diol diacetate with methanolic H_2SO_4 at room temperature when re-arrangement and hydrolysis occurred (cf. Leeds et al., 1954).

The melting point behaviour of 16α-hydroxyoestrone requires special comment. The melting point, as in the case of the urinary KC-5, was not depressed after admixture with 16-oxooestradiol-17β (m.p. 239-241.5°C.) and it seemed quite possible that at or near its melting point, 16α-hydroxyoestrone might undergo rearrangement to 16-oxooestradiol-17β.

On redetermining the melting point with a more rapid rate of heating over the range 205-230°, it was found that the previously observed shrinkage was actually a melt followed by resolidification. This suggested that the lower/

lower melting point might be the true one for 16α-hydroxyoestrone and the higher one the melting point of the rearranged product. This latter product was shown to be 16-oxooestradiol-17β.

(b) Isolation of KC-5.

Pregnancy urine (256 litres) was hydrolysed by enzyme as before and the ether-soluble phenolic ketonic fractions chromatographed on Celite on a 2 cm. diameter column in the system 70% (v/v) methanol/benzene-n-hexane (4:1 by vol.). In this way, a fraction weighing 125 mg. and containing 94 mg. of KC-5 was obtained. Pigmented and gummy impurities were removed from this material by two washings with small volumes of methanol at -20°C. and thus were obtained 64 mg. of a nearly white solid having $[\alpha]_D^{18}$ +114° (in ethanol). However, after two crystallizations from methanol, 6 mg. of material having $[\alpha]_D^{20}$ -41° (in ethanol) were obtained. In view of this failure to concentrate the dextro-rotatory component in the mixture directly, all the fractions containing KC-5 were recombined and washed once with a small volume of methanol/

methanol at -20°C. to remove pigment and gum.

The crude KC-5 (63.5 mg.), obtained in this way, was acetylated in the usual way by leaving the product overnight in 2 ml. of pyridine and 2 ml. of acetic anhydride. One crystallization from ethyl acetate-n-hexane and three from methanol yielded 15.7 mg. of a product (a) which had a melting point of 172.5-174.5°C. and $[\alpha]_D^{20}$ +146° (in ethanol). Analysis of this material gave C, 71.3%; H, 6.9%, showing good agreement with the theoretical values for a compound of the formula $C_{22}H_{26}O_5$, viz. C, 71.3; H, 7.1%.

From the material obtained by combining the mother liquors from the crystallizations of that product, a further batch of material (b), weighing 18.7 mg., was separated by washing once with a small volume of methanol at -20°C. and crystallizing once from ethyl acetate-n-hexane. This product melted from 170-173°C. and had $[\alpha]_D^{18}$ +157° (in ethanol). The mixed melting point with authentic 16α-hydroxyoestrone diacetate (m.p. 172-174.5°C.) was 170-173°C.

(c) /

FIG.17. ABSORPTION SPECTRA OF H_2SO_4 SOLUTIONS OF KC-5 DIACETATE & 16α-HYDROXYOESTRONE DIACETATE AFTER 2HRS AT 25°C.

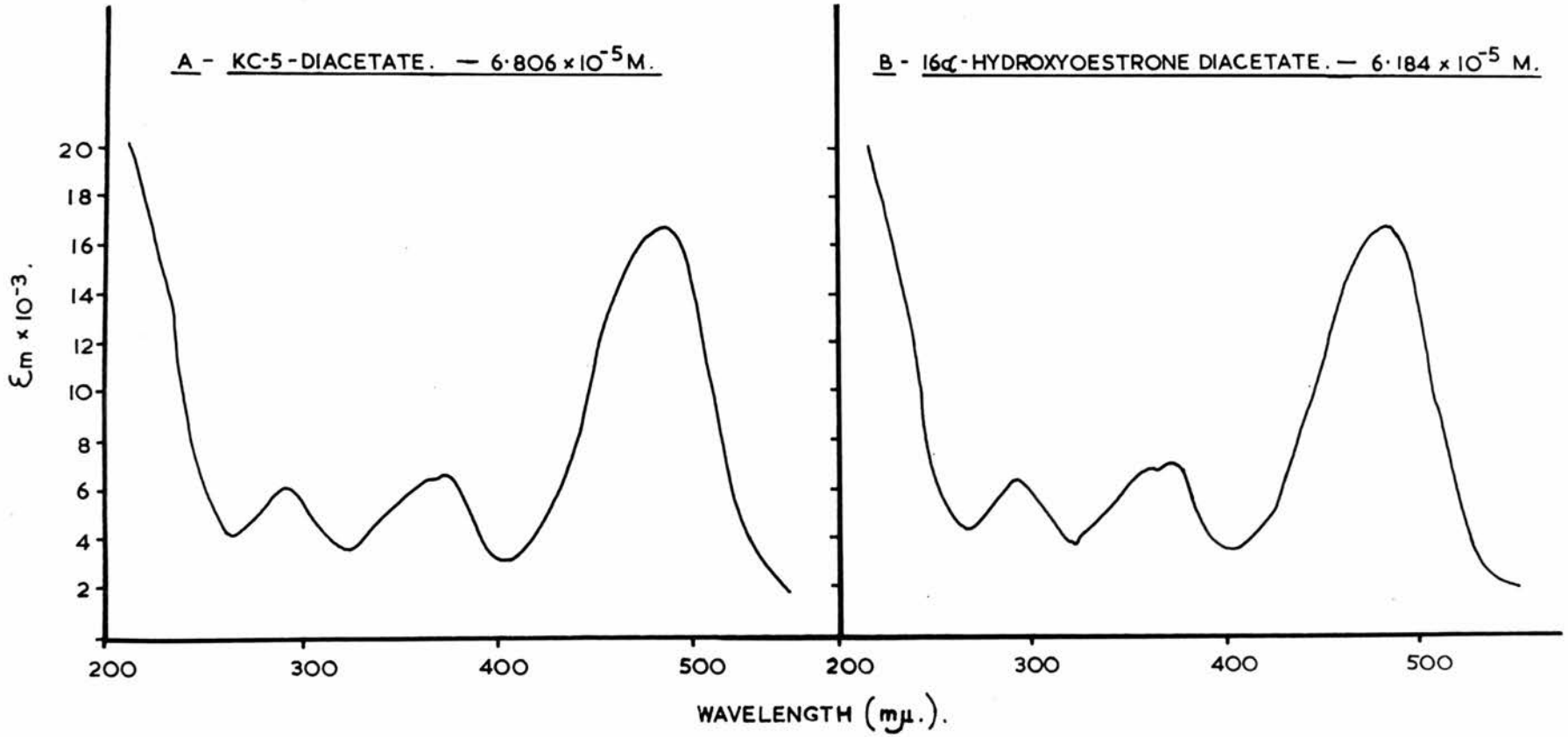
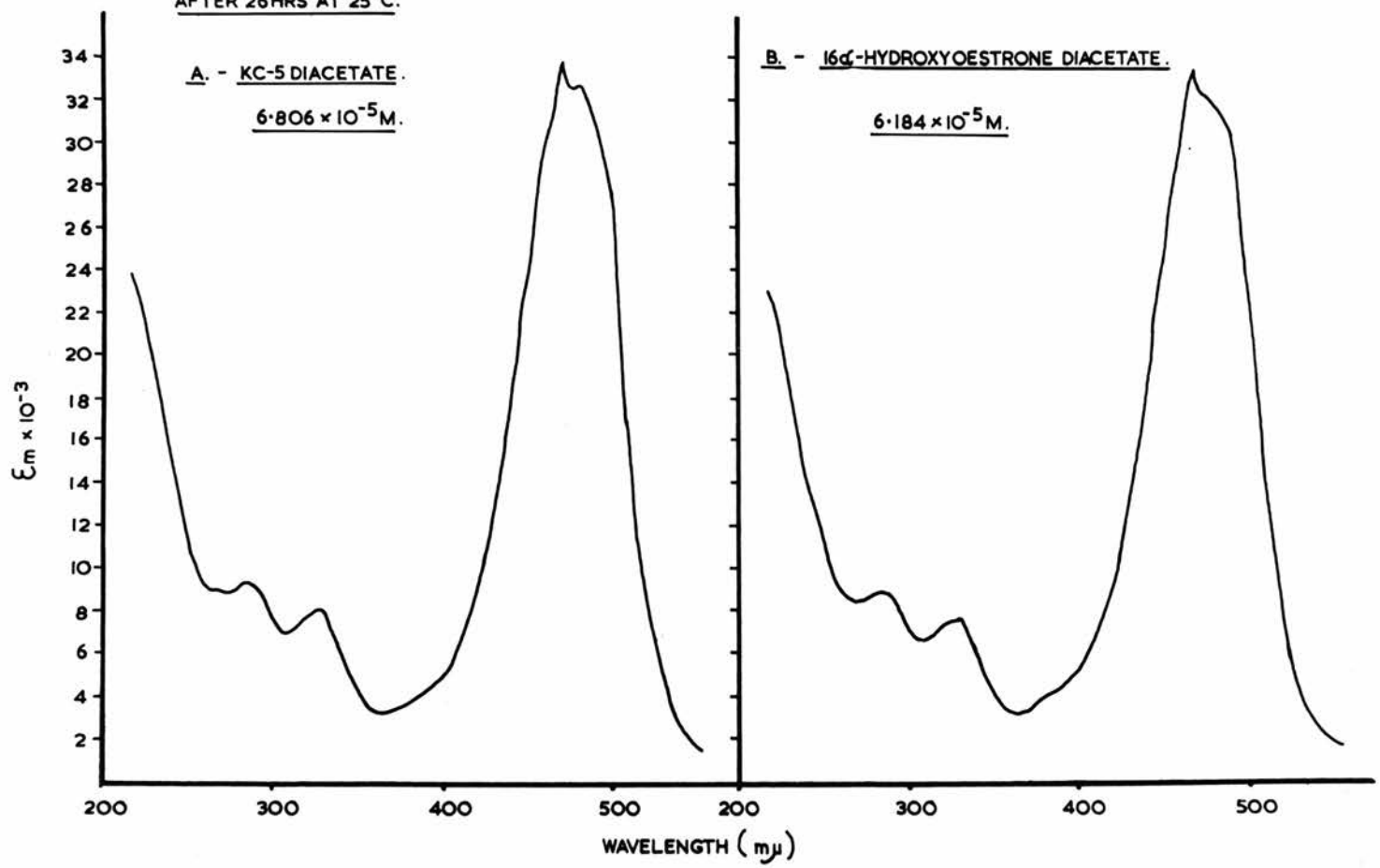


FIG.18. ABSORPTION SPECTRA OF H_2SO_4 SOLUTIONS OF KC-5 DIACETATE & 16α-HYDROXYOESTRONE DIACETATE AFTER 26 HRS AT 25°C.



(c) Sulphuric acid spectra of KC-5 diacetate and of 16α-hydroxyoestrone diacetate.

Further evidence for the identity of KC-5 diacetate was obtained by comparing the absorption spectra of solutions of KC-5 diacetate (b) and 16α-hydroxyoestrone diacetate in concentrated H_2SO_4 over the range 220-550 mμ after two hours' incubation at 25°C. The spectra were identical in all respects (Fig. 17). After a further 24 hours' incubation at 25°C., the spectra were still identical (Fig. 18).

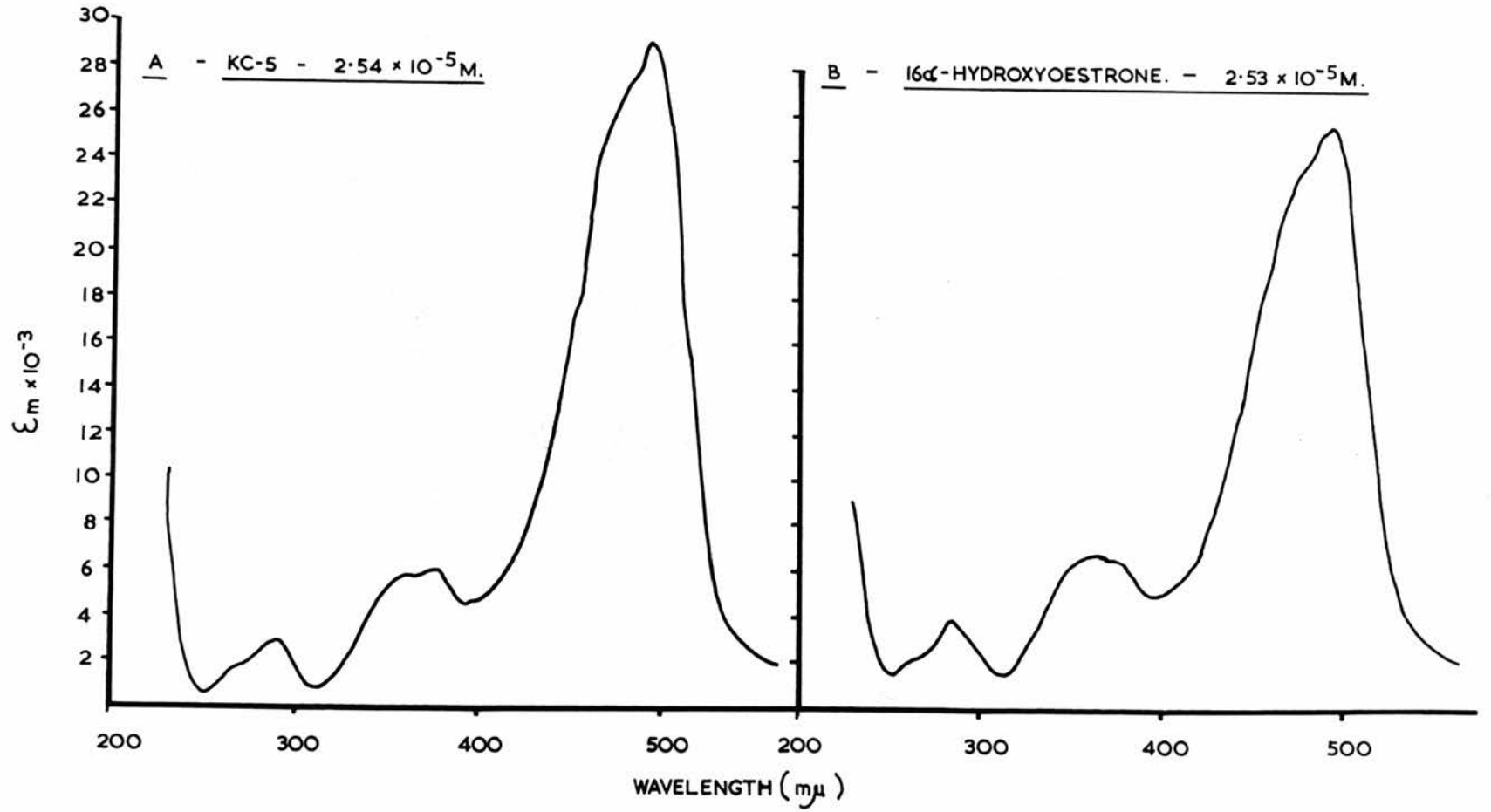
(d) Infrared spectra of KC-5 diacetate and 16α-hydroxyoestrone diacetate.

The infrared spectra of KC-5 diacetate (b) and of 16α-hydroxyoestrone diacetate, which were determined in KCl discs by Dr R.K.Callow of the National Institute for Medical Research, 'showed complete similarity in all features, both in the main bands..... and in all the bands in the fingerprint region.... .'

(e) Hydrolysis of KC-5 diacetate.

To a solution of 19.6 mg. of a mixture of batches (a) and (b) of the purified KC-5 diacetate in/

FIG. 19. ABSORPTION SPECTRA OF H_2SO_4 SOLUTIONS OF KC-5 & 16α-HYDROXYOESTRONE AFTER 2HRS AT 25°C.



in 4 ml. of methanol, was added 0.5 ml. of 5 N-H_2SO_4 and the mixture allowed to stand at room temperature for five days. After dilution with ethyl acetate, the reaction mixture was washed with 8% (w/v) $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated to dryness. The product was crystallized once from a small volume of methanol at -20°C. yielding 11.5 mg. of material having a melting point about 215°C. and 237.5-240°C. and $[\alpha]_D^{18}$ +169 (in ethanol). The mixed melting point with authentic 16α-hydroxyoestrone (m.p. about 216°C. and 238-240.5°C.) was about 217°C. and 238-240.5°C.

(f) Absorption spectra of sulphuric acid solutions of the hydrolysis product of KC-5 diacetate and of 16α-hydroxyoestrone.

A portion of the hydrolysis product was treated with 4 ml. of concentrated H_2SO_4 for two hours at 25°C. The absorption spectrum of the resultant solution closely resembled, but was not identical with that of a similarly prepared solution of 16α-hydroxyoestrone (Fig. 19). It is quite probable that these slight differences were due/

due to the rearrangement of a small amount of KC-5 to 16-oxooestradiol-17β during the hydrolysis. This same rearrangement might also explain the slightly low value for the optical rotation of the hydrolysis product of KC-5 diacetate, viz. +169° (in ethanol); cf. 16α-hydroxyoestrone $[\alpha]_D^{16}$ +180°. Unfortunately, there was insufficient of the hydrolysis product to permit of more rigorous purification.

(g) Summary.

The following is a summary of the evidence leading to the final identification of KC-5 as 16α-hydroxyoestrone.

i. The melting point of KC-5 diacetate (b) was not depressed after admixture with 16α-hydroxyoestrone diacetate.

ii. The absorption spectra of solutions of KC-5 diacetate (b) and 16α-hydroxyoestrone diacetate in concentrated H_2SO_4 over the range 220-550 mμ after two hours at 25°C. were identical.

iii./

iii. Infrared spectra of solutions of KC-5 diacetate (b) and 16α-hydroxyoestrone diacetate were identical.

iv. Hydrolysis of KC-5 diacetate gave a product whose melting point was not depressed on admixture with authentic 16α-hydroxyoestrone.

9. Conclusions

The KC-5 from pregnancy urine seems, then, to have contained 16α-hydroxyoestrone as its major component. However, 16-epioestriol was definitely shown to be present as one of the products of $NaBH_4$ reduction of the KC-5 isolated from Batches III and IV of the pregnancy urine. This could have been due to the presence in the original extract of either 16β-hydroxyoestrone or 16-oxooestradiol-17β or of both of these oestrogens. In view of work done since then by Loke (Marrian, Loke, Watson and Panattoni, 1957) on the isomerization of 16α-hydroxyoestrone, 16-oxooestradiol-17β would almost certainly have been present in the KC-5 fraction isolated from/

from urine. Loke found that treatment of 16α-hydroxyoestrone of $[\alpha]_D^{17}$ +181° (ethanol) with N-NaOH for two hours at room temperature caused the formation of a product of $[\alpha]_D^{19}$ -95° (ethanol) (cf. 16-oxooestradiol-17β $[\alpha]_D$ -89° (ethanol)) and melting point 236.5-238.5° (cf. 16-oxooestradiol-17β, m.p. 238-241°). This product on acetylation gave a substance of melting point 133-136°, this figure being hardly changed on admixture with 16-oxooestradiol-17β diacetate (m.p. 132.5-134°). During the extraction of the phenolic fraction from pregnancy urine, the 16α-hydroxyoestrone would be in contact with alkali for approximately 10 minutes. Exposure of authentic 16α-hydroxyoestrone to N-NaOH at room temperature for 10 minutes caused a drop in optical rotation from +181° to +122°, corresponding to the rearrangement of about 20% of the starting material to 16-oxooestradiol-17β.

From such evidence, it is virtually certain that there would be 16-oxooestradiol-17β present in the sample of KC-5 used for reduction, in addition to the main constituent, 16α-hydroxy-oestrone. This would also explain the rather low optical/

optical rotation of the urinary KC-5, viz. $[\alpha]_D^{15}$ +143°; cf. authentic 16α-hydroxyoestrone $[\alpha]_D^{17}$ +181°.

Loke has also produced evidence that the reduction of 16α-hydroxyoestrone by $NaBH_4$ is not so stereoselective as that of oestrone (Biel, 1951) and 16-oxooestradiol-17β (Huffman and Lott, 1955). 16α-Hydroxyoestrone diacetate always yields on reduction and subsequent hydrolysis, about 15-19% of a '16-epioestriol-like' product irrespective of the number of crystallizations to which the starting material has been subjected. Nor is the yield of this less 'polar' reduction product increased with an increased time of reduction or an increased amount of $NaBH_4$. These observations would tend to suggest that the '16-epioestriol-like' reduction product is not due to the reduction of some impurity in the starting material and that no isomerization is occurring under the alkaline conditions prevailing during the reduction. If this is so, then reduction of 16α-hydroxyoestrone diacetate must be producing a mixture of substances which on hydrolysis yield oestriol and 17-epioestriol. Also $NaBH_4$ reduction/

reduction of 16α-hydroxyoestrone itself yields about 10% of a '16-epioestriol-like' product. In view of the observation that such a product is present in rather higher yield (30%) after catalytic reduction of 16α-hydroxyoestrone in neutral ethanolic solution, it would appear to be caused not by partial rearrangement to 16β-hydroxyoestrone or 16-oxooestradiol-17β during the reduction but by non-stereoselective reduction by $NaBH_4$. Since the 17-epioestriol formed in this way behaves very similarly to 16-epioestriol in the chromatographic system used, it is quite possible, as was previously mentioned, that it was present in the less 'polar' fraction of the reduction product of KC-5 but escaped detection in subsequent chromatography. It might, however, explain the rather stepwise elution pattern observed in the chromatogram of the reduction product of urinary KC-5.

If the similar pattern in the 'oestriol' fraction is also due to the presence of two triols, then the unknown constituent must be oestra-1:3:5-triene-3:16β-17α-triol which, from

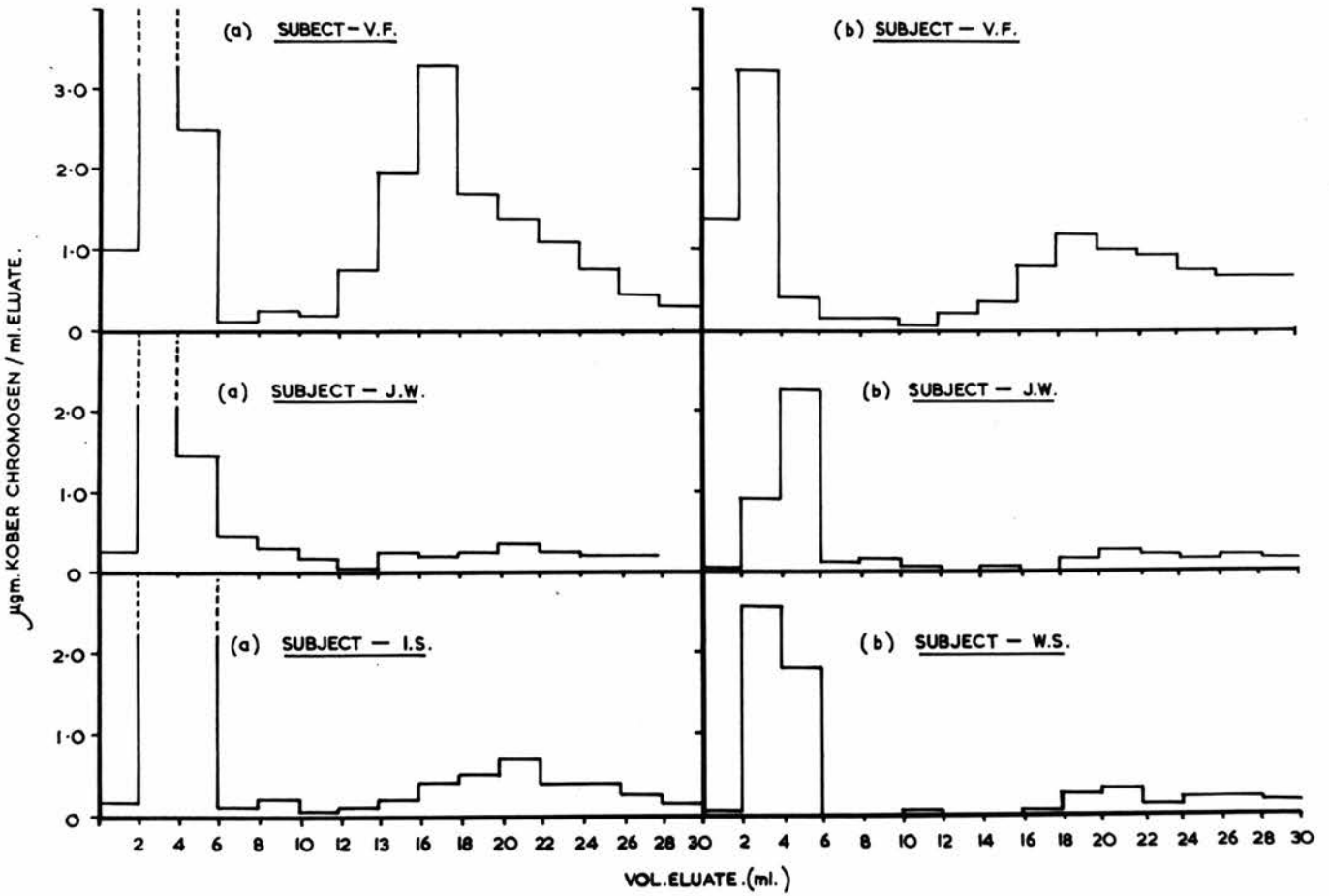
a /

a consideration of its molecular model, would be expected to be rather more 'polar' than oestriol. This triol could be produced by reduction of 16β-hydroxyoestrone if, as in the case of 16α-hydroxyoestrone, $NaBH_4$ reduction is not completely stereoselective. 16-Epioestriol would be produced at the same time. In view of this it is hoped to attempt to detect 16β-hydroxyoestrone in the mother liquors remaining after isolation of 16α-hydroxyoestrone.

There remains the possibility that the 16-epioestriol produced by reduction of urinary KC-5 arose from 16-oxooestradiol-17β which was actually present in the urine fraction before treatment with alkali. If this were so, then from a consideration of the optical rotation of the urinary KC-5, it must have been only a very minor constituent. Recently, Levitz, Spitzer and Twombly (1956) have claimed to have detected the presence of radioactive 16-oxooestradiol-17β in the urine of human subjects after the administration of oestradiol-17β - 16 ^{14}C. At the moment, it would seem advisable to treat this claim with reserve.

10. /

FIG.20. REPRESENTATIVE PARTITION CHROMATOGRAMS OF THE PHENOLIC KETONIC FRACTIONS OF MENSTRUAL CYCLE URINES IN SYSTEM 70% (V/V) METHANOL / BENZENE-n-HEXANE. (4:1. BY VOL.) DURING (a) FOLLICULAR PHASE (b) LUTEAL PHASE.



10. A Preliminary Search for 16α-Hydroxyoestrone in Menstrual Cycle Urines of the Follicular and Luteal Phases.

Twenty-four hour samples of urine, collected from apparently normal young women during the follicular and luteal phases of the menstrual cycle, were hydrolysed by enzyme and the ether-soluble phenolic ketonic fraction separated from each as described in Section III,2. This fraction was chromatographed on a micro column with the system 70% (v/v) methanol/benzene-n-hexane (4:1 by vol.).

Altogether eight follicular phase urines and seven luteal phase urines from eight different women were analysed. With only two exceptions, these urine fractions contained a Kober chromogen which was eluted from the column in the fraction from 16-32 ml. Representative elution patterns are shown in Fig. 20. In five subjects, this Kober chromogen was excreted in slightly higher amounts during the follicular phase than during the luteal phase. One subject (V.F.) excreted 25 μg./24 hours during the follicular phase and 15 μg./24 hours during the luteal phase. These amounts/

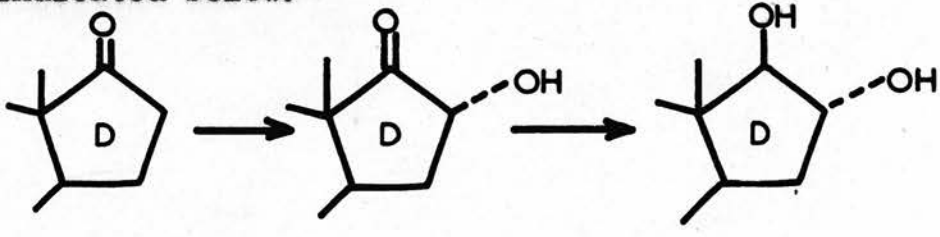
amounts, however, were very much in excess of the excretion of this Kober chromogen by the other subjects, the general level of excretion being 1-7 μg./24 hours during the follicular phase and 1-3 μg./24 hours during the luteal phase.

It must be emphasized, however, that these determinations can be regarded as no more than very preliminary evidence that 16α-hydroxyoestrone may be excreted during these phases of the cycle. The method does not claim to be quantitative and no attempt was made to establish the specificity of the method. It is hoped to have available soon a method which can be relied upon to be reasonably quantitative.

IV. GENERAL DISCUSSION

In view of the isolation of 16α-hydroxyoestrone from human pregnancy urine and the preliminary evidence for its excretion during certain phases of the menstrual cycle, it seems fitting at this stage to postulate its possible role in the metabolism of oestrogens.

My colleague, Dr J.B.Brown has carried out experiments in which he injected 16α-hydroxyoestrone into men and postmenopausal women (personal communication). He detected in the urine of these subjects an amount of oestriol equivalent to about 40% of the injected oestrogen. It is hoped that further work in this field will soon be carried out but, meanwhile, the most probable sequence of reactions seems to be as indicated below.

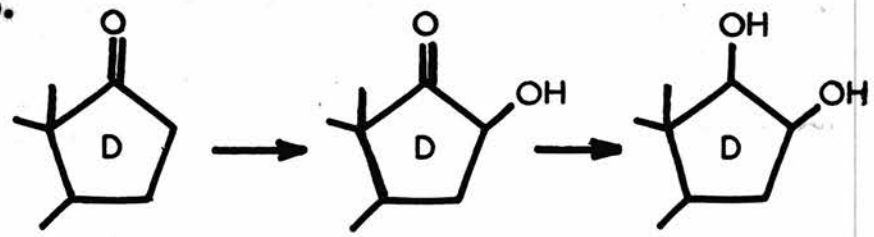


i.e. oestrone is hydroxylated to yield 16α-hydroxyoestrone which is subsequently reduced to oestriol.

By /

By analogy, 16-epioestriol might be formed by a similar pathway involving 16β-hydroxylation of oestrone followed by reduction of the C17 ketonic group.

i.e.



So far, however, 16β-hydroxyoestrone has not been detected in urine. A sample of 16β-hydroxyoestrone diacetate was prepared by Mr K.H.Loke by the action of lead tetraacetate on oestrone enol acetate, according to the method used by Johnson, Gastambide and Pappo (private communication to Professor Marrian) for the preparation of 16-epiacetoxy-androsterone acetate. This sample was hydrolysed and when chromatographed on the usual micro column was found to behave very similarly to 16α-hydroxyoestrone and 16-oxo-oestradiol-17β. It is to be expected then that if 16β-hydroxyoestrone is excreted during pregnancy, it will be concentrated in the KC-5 fractions. It will be interesting to try to detect it in the mother liquors remaining after the/

the separation of 16α-hydroxyoestrone. It is also to be expected that if 16β-hydroxyoestrone is excreted, its concentration will be very much lower than that of 16α-hydroxyoestrone, since 16-epioestriol is excreted and presumably formed in the body, in amounts much less than those of oestriol.

Neither of the metabolic routes suggested above, however, include 16-oxooestrone which was isolated in crystalline form by Serchi (1953). This was achieved from an extract of only ten litres of menstrual cycle urine. Slaunwhite and Sandberg (1956) also detected 16-oxooestrone in the urine of one woman to whom they had administered oestrone-16 ^{14}C during the luteal phase of the menstrual cycle. However, similar injections to two other women at other stages in the cycle failed to cause the excretion of labelled 16-oxooestrone in the urine.

Levitz, Spitzer and Twombly (1956) administered oestradiol-17β-16 ^{14}C to women and analysis of the urine indicated the presence of labelled 16-oxooestradiol-17β. If this oestrogen is a true constituent of urine, then it should have been present in KC-5 concentrates obtained/

obtained from pregnancy urine. These fractions did contain a small proportion of laevorotatory 16-oxooestradiol-17β but the amount could easily have been accounted for by the ease of isomerization of 16α-hydroxyoestrone to 16-oxooestradiol-17β during the time that the phenolic fraction was in contact with alkali. It is also extremely unlikely that any 16-oxooestradiol-17β originally present in the extract would have been converted to 16α-hydroxyoestrone, since treatment of authentic 16-oxooestradiol-17β by heating it to its melting point, by subjecting it to a Girard reaction and to partition chromatography gave no evidence of any isomerization. It would therefore appear that, if 16-oxooestradiol-17β is actually excreted in urine, it must be present in extremely small amounts.

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She is also indebted to the staff of the Simpson Maternity Pavilion, the Elsie Inglis Maternity Hospital, Dr A. Klopper of the Clinical Endocrinology Research Unit (Medical Research Council) and to various members of the Department of Biochemistry for their cooperation in the collection of urine.

APPENDIX I.

APPENDIX I.

Preparation of Materials

1. Oestrogen samples

(a) Oestriol

The preparation described by Marrian and Bauld (1955) was used. It had a melting point of 279-280° and $[\alpha]_D^{19}$ +61° (ethanol). It yielded a triacetate of melting point 126-128°.

(b) 16-Epioestriol

This was prepared from oestrone by the method described by Huffman and Darby (1944). It was crystallized twice from aqueous methanol, treated with charcoal in hot methanolic solution, and finally recrystallized once from methanol-benzene. Its melting point was 284-285° and it had $[\alpha]_D^{15}$ + 76° (ethanol). It yielded a triacetate of melting point 151-153°.

(c) 16-Oxooestradiol-17β

This was prepared from oestrone by the method of Huffman (1942) and Huffman and Lott (1948) and was crystallized once from 50% (v/v) aqueous acetic acid and once from dilute methanol. The melting point was 239-241° and it had $[\alpha]_D^{12}$ -89° (ethanol). It yielded a diacetate of melting point 132.5-134°.

(d) 16α-Hydroxyoestrone was prepared as described in Section III,8.

(e) 17-Epioestriol

A sample of authentic 17-epioestriol was kindly supplied by Dr L. Ruzicka of Zurich.

2. Solvents

Ether. A.R. quality ether was always used and was tested immediately before use for the presence of peroxides. This was done by shaking 3 ml. of the ether with 2 ml. of 1% KCNS containing a few drops of $FeSO_4$. If no pink colour developed, the ether was used without further treatment. If, however, peroxides were present, the ether was purified by shaking several times with saturated $FeSO_4$, washing with water, drying over $NaSO_4$ and redistilling.

Benzene. Benzene was purified by preliminary distillation, washing with tap water continuously for three days or thorough shaking ten times with one fifth of its volume of water, drying over Na_2SO_4 and finally redistilling.

n-Hexane. n-Hexane was washed twice with one fifth of its volume of concentrated H_2SO_4 and six times with one fifth of its volume of water. After/

After drying over Na_2SO_4, the n-hexane was redistilled.

Ethanol and Methanol. Each of these solvents was heated under reflux for 15-20 hours with 5% (w/v) NaOH and 5% (w/v) zinc dust, then distilled twice.

Ethylene dichloride. Ethylene dichloride was purified by washing twice with one fifth of its volume of N-NaOH and then with water until neutral. After drying over Na_2SO_4, the solvent was distilled, then redistilled shortly before use.

Chloroform. Chloroform of B.P. standard was distilled once.

Acetone. Acetone used in the preparation of β-glucuronidase from Patella vulgata was used without any purification.

Ethyl acetate. Ethyl acetate was washed once with one tenth of its volume of 5% (w/v) Na_2CO_3, once with one tenth of its volume of saturated $CaCl_2$, thoroughly washed with water, dried over $CaCl_2$ and distilled.

3. /

3. Celite. Celite 535 (Johns Manville & Co., Ltd. London) was purified by partially calcining at 400° for 4 hours, leaving it in 10 N-HCl overnight, washing with water until free of Cl^- and Fe^{+++} and drying. It was stored until use in an oven at 120°.

4. Chemicals. All chemicals used were A.R. quality with the exception of NaCl used in large-scale isolation work. The latter preparation was B.P. quality.

5. Buffers.

pH 10.5 buffer was prepared by making up 52 ml. of 5 N-NaOH to 400 ml. with 8.5% (w/v) $NaHCO_3$.

pH 12.4 buffer was prepared by mixing 250 ml. of 0.1 N-Na_2HPO_4 with 330 ml. of 0.1 N-NaOH and 100 ml. of water.

APPENDIX II.

PUBLICATIONS.

[From THE BIOCHEMICAL JOURNAL, Vol. 63, No. 1, pp. 64–69, 1956]
 PRINTED IN GREAT BRITAIN

Observations on the Occurrence of 16-*epi*oestriol in Urine

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Department of Biochemistry, University of Edinburgh

(*Received* 14 *November* 1955)

16-*epi*Oestriol (oestra-1:3:5-triene-3:16β:17β-triol) was recently isolated from the urine of pregnant women by Marrian & Bauld (1954, 1955). However, the yield was small and the isolation procedure was somewhat rigorous, and the possibility of the isolated material being an artifact could not therefore be excluded. The authors considered the possibility, not specifically mentioned, that epimerization of the C-16 hydroxyl group of oestriol might have occurred to a small extent, either during the preliminary hot acid hydrolysis of the urine, or subsequently through the use of aqueous alkali in the fractionation of the urinary extract. One of the purposes of the work reported here was to investigate this possibility.

This investigation involved the development of a method for the rough determination of 16-*epi*oestriol in urine extracts; and such a method being in due course available, a preliminary study has been made of the urinary excretion of 16-*epi*oestriol during the menstrual cycle.

EXPERIMENTAL

Materials

Oestriol. The preparation described by Marrian & Bauld (1955) was used. It had a corrected m.p. of 284·5–286°.

16-epi*Oestriol.* This was prepared from oestrone by the method of Huffman & Darby (1944). It was crystallized twice from aqueous methanol, treated with charcoal in hot methanolic solution, and finally recrystallized once from methanol–benzene. It melted at 289–291° (corr.) and had $[\alpha]_D^{15}$ +76° (0·297 % in ethanol).

β-Glucuronidase preparation. This was a water-soluble preparation obtained from *Patella vulgata* by the method of Dodgson & Spencer (1953). It contained 1 500 000 units of β-glucuronidase activity/g. determined by the method of Fishman (1948) with phenolphthalein glucuronide as substrate. Such preparations from *Patella vulgata* also contain sulphatases.

Miscellaneous. Benzene was purified by a preliminary distillation, washing continuously with tap water for 3 days, drying over anhydrous Na_2SO_4, and finally redistilling. Ethylene dichloride and methanol were purified as described by Bauld (1955). Chloroform was B.P. purity and was distilled once. *n*-Hexane was washed in a separating funnel twice with conc. H_2SO_4 and six times with water. After drying over anhydrous Na_2SO_4 it was distilled. Ethanol was purified as described by Bauld (1954). Celite 535 (Johns Manville and Co. Ltd., London) was purified as described by Bauld (1955). The 'pH 12·4 buffer' was prepared by mixing 250 ml. of 0·1 N-Na_2HPO_4 with 330 ml. of 0·1 N-NaOH and 100 ml. of water. The 'pH 10·5 buffer' was prepared as described by Marrian & Bauld (1955).

Methods

Analytical partition chromatograms. The procedures used were based on those of Bauld (1955), 1 × 10 cm. columns being used with 5 ml. of stationary phase on 5 g. of Celite 535. 'Channelling' due to uneven packing of the columns was avoided by rotating the columns during packing. Solutes were applied to the column dissolved in 2 ml. of mobile phase, and, when the solution had entered the column completely, elution with mobile phase at a percolation rate of 10–12 ml./hr. was commenced. A constant temperature of 15–16° was maintained. Successive 1 ml. or 2 ml. portions of the eluates were collected, evaporated in a boiling-water bath under a stream of air, and the Kober reaction as described below, carried out on the residues.

Kober reaction. The method of Brown (1952) as modified by Bauld (1954) was employed, the 'oestriol reagent' of the latter being used.

RESULTS

Determination of 16-epi*oestriol in urine*

The method developed for the rough determination of 16-*epi*oestriol in urine was a modification of that of Bauld (1953) for the determination of oestriol.

Preliminary experiments with oestriol and 16-*epi*oestriol showed that, in the benzene–water partition of the Bauld method, about 95 % of the oestriol present is in the aqueous phase, while there is rather less than 60 % of 16-*epi*oestriol because of its relatively lower 'polarity'. However, the proportion of 16-*epi*oestriol in the aqueous phase could be raised to about 85 % by doubling the proportion of water used in the partition. About 8 % of any oestradiol-17β present was found to pass into the aqueous phase, but was not found to interfere in the subsequent separation of the 16-*epi*oestriol fraction on the chromatograms.

It seemed likely that in the determination of 16-*epi*oestriol in pregnancy urines the relatively large amounts of oestriol present might interfere in the separation of the 16-*epi*oestriol fractions in the chromatograms. However, this potential difficulty could be overcome by washing the final ether extract obtained before chromatography with a pH 12·4 phosphate buffer. By this means about 70–90 % of the oestriol present could be removed, with little loss of 16-*epi*oestriol.

The complete procedure developed for the determination of 16-*epi*oestriol in urine was as follows:

For urines from normal menstruating women determinations were carried out on one-half of the 24 hr. specimen, and for urines of pregnant women on one-tenth of the 24 hr. specimen diluted to 500 ml. with water. After hydrolysis with acid or with β-glucuronidase the urine was extracted four times with about one-quarter volumes of ether, and the combined extract washed once with 100 ml. of pH 10·5 buffer. The extract was then shaken vigorously for a few minutes with 25 ml. of 2 N-NaOH, and after the addition of 100 ml. of 8·5 % (w/v) $NaHCO_3$ solution, the mixture was shaken again and the aqueous layer discarded. After further washing, once with 25 ml. of 8·5 % (w/v) $NaHCO_3$, and with 3 × 12 ml. of water, the extract was evaporated to dryness.

The residue was dissolved in 1·5 ml. of ethanol and the solution quantitatively transferred to a separating funnel with 25 ml. of benzene. The benzene solution was extracted twice with 50 ml. and twice with 25 ml. portions of water. After the addition of 15 ml. of 10 N-NaOH, the combined aqueous extract was boiled under reflux for 30 min., cooled, neutralized to phenolphthalein by passing CO_2 into the solution, and extracted four times with 50 ml. portions of ether.

With samples of urine from pregnant women the ether extract was washed twice with 40 ml. portions of pH 12·4 buffer, the buffer washing back-extracted once with 40 ml. of ether, and the

combined ether extracts were washed twice with 24 ml. portions of water and evaporated to dryness.

With samples of urine from the menstrual cycle the extract was not washed with buffer, but once with 12 ml. of 8·5 % (w/v) $NaHCO_3$ and twice with 6 ml. portions of water before being evaporated to dryness.

The residue was chromatographed by using the system 70 % methanol–ethylene dichloride, and Kober chromogens were determined on successive 1 or 2 ml. portions of the eluate.

With extracts of pregnancy urines good separation of the 16-*epi*oestriol fraction (6–12 ml.) was obtained in the chromatograms (Fig. 1); although no claim is made that the whole procedure is quantitatively satisfactory when applied to urines of pregnancy, it is believed that it is specific for 16-*epi*oestriol and that it provides a reliable rough method of assessment which is of value for comparative purposes. On the other hand, the chromatographic separation of 16-*epi*oestriol fractions from extracts of urines collected from women during the menstrual cycle was much less clear-cut (Fig. 2), and of necessity therefore the possibility had to be considered that the procedure might not be specific for 16-*epi*oestriol when applied to such urines.

Evidence against 16-epi*oestriol being an artifact*

Hot acid treatment of oestriol in aqueous solution. Three 3·0 ml. portions of an ethanolic solution of oestriol containing 50 μg./ml. were added respectively to three 500 ml. portions of water. After heating the three solutions to boiling under reflux 75 ml. of conc. HCl (A.R.) was added to each, and the boiling continued for 1, 2 and 4 hr. respectively. An untreated 'control' solution was also prepared by adding 3·0 ml. of the ethanolic solution of oestriol to 575 ml. water. Each of the four solutions was then extracted once with 150 ml. and three times with 125 ml. ether; and in each case the combined ether extract was washed three times with 50 ml. portions of 8·5 % (w/v) $NaHCO_3$, and three times with 50 ml. portions of water. The washed ether solutions were evaporated to dryness and the residues chromatographed by using the system 70 % methanol–ethylene dichloride. Kober chromogens were determined in successive 2 ml. portions of the eluates. The recoveries of oestriol in the three 'acid hydrolysis' experiments compared with that in the control experiment were 89, 91 and 88 % respectively, and in no case was any Kober chromogen less 'polar' than oestriol detected. It was concluded that oestriol is not epimerized to 16-*epi*oestriol to any significant extent on boiling in aqueous solution with 15 vol. % of conc. HCl.

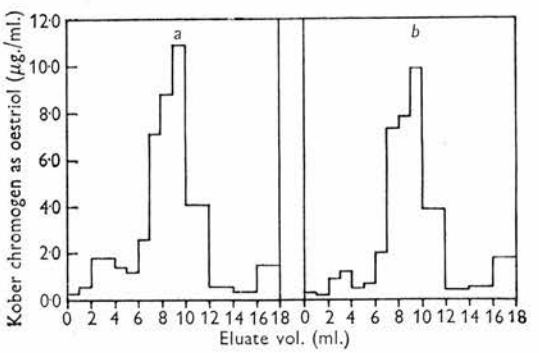

Fig. 1. Partition chromatograms (70 %, v/v, methanol–ethylene dichloride) on extracts from a sample of late-pregnancy urine after (*a*) hydrolysis with β-glucuronidase (*Patella vulgata*) and (*b*) hot acid hydrolysis.

Treatment of oestriol with alkali in aqueous solution. To investigate the possibility that epimerization of oestriol to 16-*epi*oestriol might occur through exposure to aqueous alkali under the conditions used in the quantitative procedure of Bauld (1953) or in the procedure for the isolation of 16-*epi*oestriol as used by Marrian & Bauld (1955) the following experiment was carried out:

Each of two solutions containing 75 μg. of oestriol in 575 ml. of water was extracted once with 150 ml. and three times with 125 ml. ether. Each of the combined ethereal extracts was washed once with 100 ml. of pH 10·5 buffer, shaken with 25 ml. of 2N-NaOH, and, after the addition of 100 ml. of 8·5 % (w/v) $NaHCO_3$, shaken again and the aqueous layer discarded. The ether solutions were further washed once with 25 ml. of 8·5 % (w/v) $NaHCO_3$ and three times with 12 ml. water, and evaporated to dryness. The residues were each dissolved in 150 ml. of water and boiled under reflux for 30 min. after the addition of 15 ml. of 10N-NaOH. Carbon dioxide was passed into the solutions until they were acid to phenolphthalein, and the solutions then extracted four times with 50 ml. portions of ether. The combined ethereal extracts were each washed once with 12 ml. of 8·5 % (w/v) $NaHCO_3$ and twice with 6 ml. of water, and evaporated to dryness. The residues were chromatographed with the system 70 % (v/v) methanol–ethylene dichloride.

In each case the recovery of oestriol was only 71 %, but no trace of any Kober chromogen less 'polar' than oestriol could be detected. It was concluded that treatment with aqueous alkali, as employed in the procedure for the purification of urine extracts, does not result in the formation of significant amounts of 16-*epi*oestriol by the epimerization of oestriol.

Comparison of yields of 16-epi*oestriol from pregnancy urine after hot acid hydrolysis with those obtained after hydrolysis with β-glucuronidase.*

Although it had been shown that prolonged treatment of oestriol in aqueous solution with hot acid results in no detectable formation of 16-*epi*oestriol, the possibility still remained that oestriol conjugates in urine might be slightly epimerized during hydrolysis with hot acid. To investigate this possibility the yield of 16-*epi*oestriol obtained from pregnancy urine after the usual hot acid hydrolysis was compared with that obtained after incubation with a β-glucuronidase preparation from *Patella vulgata*.

Experiments were carried out on four different 24 hr. urine specimens collected during late pregnancy. The specimens were diluted to 2500 ml. and two 250 ml. samples removed from each for acid hydrolysis. After dilution of these to 500 ml. they were hydrolysed in the usual manner by boiling for 60 min. after the addition of 15 vol. % of conc. HCl. Two further 250 ml. samples were removed for the enzymic hydrolysis. After adjustment of these to pH 4·7 with acetic acid 25 ml. of M acetate buffer (pH 4·7) and 385 mg. of the enzyme preparation were added to each, and the mixture was incubated for 48 hr. at 37°. Each incubation mixture was finally diluted to 500 ml. with water.

16-*epi*Oestriol was determined in the two sets of samples thus obtained. The results, which are shown in Table 1, require some comment. The fact that in each case enzymic hydrolysis yielded 16-*epi*oestriol in amounts which were of the same order as those obtained after acid hydrolysis disposes of the possibility that 16-*epi*oestriol is formed by the epimerization of oestriol conjugates during hot acid hydrolysis. In three instances the yields of 16-*epi*oestriol obtained after enzymic hydrolysis were somewhat lower than those obtained after acid hydrolysis. This, however, is not surprising, since troublesome emulsions were formed during the extraction of the urines after hydrolysis by the enzyme, and may have resulted in appreciable losses.

Urinary excretion of 16-epi*oestriol during the menstrual cycle*

Preliminary experiments. To investigate the possibility that 16-*epi*oestriol might be excreted in the urine during the menstrual cycle six 24 hr. specimens of urine were collected from two normal young women. Three of these samples were obtained a few days before the expected time of ovulation during the follicular phase of oestrogen secretion, and three about midway through the second half of the cycle during the luteal phase of oestrogen secretion (cf. Brown, 1955).

Each urine specimen was divided into two equal portions and 16-*epi*oestriol was determined in each after hydrolysis with acid in the usual manner.

Representative elution patterns obtained in the chromatograms are shown in Fig. 2. It will be seen that whereas in each case a Kober chromogen was eluted over the range 6–12 ml., the elution pattern was considerably confused by the relatively large amounts of chromogenic material eluted near the solvent front. In these circumstances it was thought that little quantitative significance could be attached to these results; indeed the possibility had necessarily to be considered that this fraction might partly or entirely consist of chromogens other than 16-*epi*oestriol.

*Identification of the '*16-epi*oestriol-like' Kober chromogen excreted during the menstrual cycle*

To obtain further evidence on the identity of the '16-*epi*oestriol-like' Kober chromogen excreted during the menstrual cycle, fourteen and ten 24 hr. urine specimens were collected during the follicular and luteal phases respectively from four different normal young women. Each sample was separately hydrolysed with acid, extracted and fractionated by the methods previously described,

Table 1. *Comparison of yields of* 16-epi*oestriol from pregnancy urine after hydrolysis with acid with those after hydrolysis with β-glucuronidase* (Patella vulgata)

Urine specimen	16-*epi*Oestriol/24 hr. urine (μg.) After acid hydrolysis	After enzymic hydrolysis
1	458, 442	445, 418
2	572, 572	352, 397
3	814, 788	628, 530
4	320, 327	425, 406

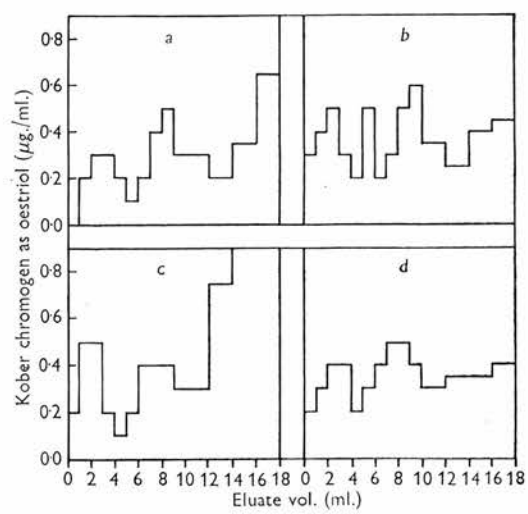

Fig. 2. Partition chromatograms (70%, v/v, methanol–ethylene dichloride) on extracts obtained from menstrual-cycle urines. (*a*) and (*b*) Follicular phase; (*c*) and (*d*) luteal phase.

and finally chromatographed with the system 70 % methanol–ethylene dichloride. The fractions eluted from the columns between 6 and 12 ml. from the fourteen follicular-phase specimens were combined in one 'pool', and those from the ten luteal-phase specimens combined in another. Each 'pool' was then divided into three equal portions.

One portion from each pool was chromatographed with the system 70 % (v/v) methanol/benzene–ethylene dichloride (3:1, by vol.), while second portions were chromatographed with the different system 50 % (v/v) methanol/hexane–chloroform (3:1, by vol.). For comparison authentic 16-*epi*-oestriol was chromatographed in each of these systems. The results (Fig. 3) showed that in both of these solvent systems the chromatographic behaviour of the '16-*epi*oestriol-like' Kober chromogens closely resembled that of authentic 16-*epi*-oestriol.

The third portions from each pool were each methylated as follows: The material was dissolved in 50 ml. of 0·4 N-NaOH, and, after warming to 37°, 1 ml. of methyl sulphate (redistilled) was added, the mixture shaken and maintained at 37° for 30 min. 5 N-NaOH (2 ml.) and a further 1 ml. of methyl sulphate were then added, and after shaking for a few minutes, the mixture was maintained at 37° for a further 45 min. After cooling the mixture was extracted once with 100 ml. of ether, and the extract washed four times with 25 ml. portions of water and evaporated to dryness. For comparison 25 μg. of authentic 16-*epi*oestriol was methylated under identical conditions.

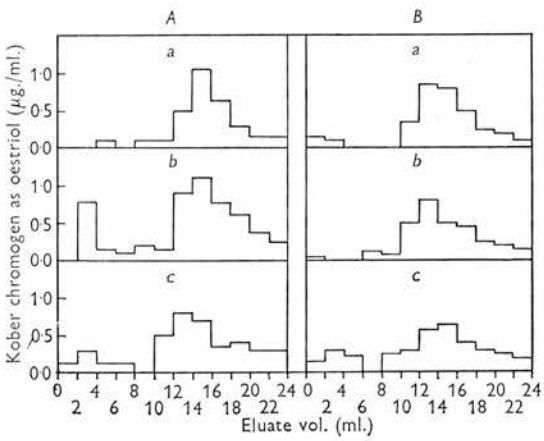

Fig. 3. Chromatographic identification of '16-*epi*oestriol-like' fractions (ELF) from menstrual-cycle urines. *A*, 70 % (v/v) methanol/benzene–ethylene dichloride (3:1, by vol.). *B*, 50 % (v/v) ethanol/*n*-hexane–chloroform (3:1, by vol.). (*a*) Authentic 16-*epi*oestriol; (*b*) ELF from follicular-phase urine; (*c*) ELF from luteal-phase urine.

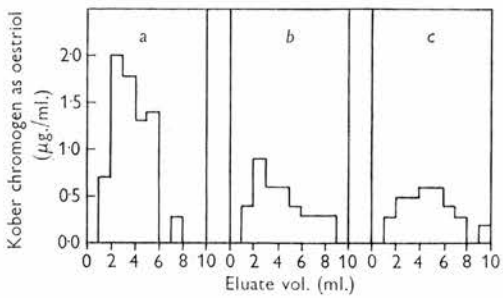

Fig. 4. Chromatographic identification of '16-*epi*oestriol-like' fractions (ELF) from menstrual-cycle urines [70 % (v/v) methanol/benzene–*n*-hexane (1:1, by vol.)]. (*a*) Methylated 16-*epi*oestriol; (*b*) methylated ELF from follicular-phase urine; (*c*) methylated ELF from luteal-phase urine.

The methylated products were chromatographed with the system 70 % methanol/benzene–*n*-hexane (1:1, by vol.). The results (Fig. 4) showed that in this solvent system the chromatographic behaviour of the methylated urinary '16-*epi*-oestriol-like' Kober chromogens closely resembled that of methylated 16-*epi*oestriol.

DISCUSSION

The evidence presented in this paper shows that oestriol does not give rise to any chromatographically detectable amounts of 16-*epi*oestriol either by prolonged boiling in aqueous solution in the presence of HCl or by alkali treatment as employed in the fractionation of urinary extracts. Furthermore, it has been shown that the yields of 16-*epi*oestriol which can be obtained from the urine of pregnancy after hydrolysis with β-glucuronidase are of the same order of magnitude as those obtained after acid hydrolysis of the urine. It can therefore be concluded with reasonable certainty that 16-*epi*oestriol occurs as such in pregnancy urine, and that the material previously isolated from this source by Marrian & Bauld (1955) was not an artifact. It is noteworthy that the concentrations of 16-*epi*oestriol in late-pregnancy urine as determined in these experiments (0·35–0·80 mg./24 hr.) are considerably higher than would have been expected from the results of Marrian & Bauld (1955), who isolated 16-*epi*-oestriol in a yield of about 0·1 mg./l.

Evidence has been presented to show that a '16-*epi*oestriol-like' Kober chromogen is excreted in the urine during both the follicular and luteal phases of the menstrual cycle. Since the chromatographic behaviour of this Kober chromgen resembles that of authentic 16-*epi*oestriol in three different partition solvent systems, and since that of the methylated Kober chromogen resembles that of

methylated 16-*epi*oestriol it has been concluded that the Kober chromogen is identical with the latter. The amounts of 16-*epi*oestriol found in the pooled extracts from both follicular phase (14th–16th day) and luteal phase (22nd–24th day) urines were equivalent to about 1 μg./24 hr. urine volume. It is not unlikely that the true figure may be considerably higher than this.

SUMMARY

1. Evidence is presented which indicates that the 16-*epi*oestriol isolated by Marrian & Bauld (1955) from the urine of pregnant women was not an artifact formed by the epimerization of oestriol.

2. A method, based on that of Bauld (1953) for urinary oestriol, is described which permits of the rough determination of 16-*epi*oestriol in pregnancy urine.

3. The amounts of 16-*epi*oestriol determined in late-pregnancy urine after hot acid hydrolysis or after hydrolysis with β-glucuronidase (*Patella vulgata*) were within the range of 350–800 μg./24 hr. urine volume.

4. A '16-*epi*oestriol-like' Kober chromogen has been detected in urine specimens collected from the 14th to 16th and from the 22nd to 24th days of the menstrual cycle in amounts equivalent to about 1 μg./24 hr. urine volume.

5. Chromatographic evidence is presented which indicates that this Kober chromogen is identical with 16-*epi*oestriol.

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The Detection of 16-Oxooestradiol-17β in the Urine of Pregnant Women. By ELIZABETH J. D. WATSON and G. F. MARRIAN. (*Department of Biochemistry, University of Edinburgh*)

Following the isolation of 16-*epi*oestriol from the urine of pregnant women, Marrian & Bauld (1955) tentatively suggested that the common metabolic precursor of both *epi*oestriol and oestriol might be 16-oxooestradiol-17β (cf. Huffman & Grollman, 1947). A systematic search for 16-oxooestradiol-17β in the urine of pregnant women has now been conducted.

Ether-soluble 'neutral and phenolic' fractions from acid-hydrolysed pregnancy urine were treated with Girard's Reagent T by refluxing in ethanol-acetic acid solution. From the ketonic fractions thus obtained the phenolic-ketonic fractions were separated in the usual way. Such phenolic-ketonic fractions were partitioned on Celite columns with 70% methanol as stationary phase and benzene-ethylene dichloride (75:25) as mobile phase.

These chromatograms revealed the presence in the urinary extracts of two different ketonic Kober-chromogens. The elution pattern of the less 'polar' of these was indistinguishable from that of oestrone in the same solvent system, while the elution pattern of the more 'polar' one was indistinguishable from that of synthetic 16-oxo-oestradiol-17β prepared by the method of Huffman & Lott (1948). In three experiments on different samples of late pregnancy urine the yields obtained of the 'more polar' Kober-chromogen were 81, 307 and 307 μg./24 hr. respectively.

The phenolic-ketonic fraction from 200 l. of late pregnancy urine was subjected to a 10-transfer countercurrent distribution in separating funnels using the solvent system 50% ethanol-benzene: hexane (80:20). The observed distribution of Kober-chromogens closely resembled the theoretical distribution for a mixture of oestrone and 16-oxooestradiol-17β which was calculated from the determined partition ratios of these compounds (oestrone, K = 9·8; 16-oxooestradiol-17β, K = 0·95).

In view of this evidence it is believed that the 'more polar' ketonic Kober-chromogen is identical with 16-oxooestradiol-17β.

Yields of 16-oxooestradiol-17β from late pregnancy urine were increased 2- to 3-fold by substituting incubation at pH 4·6 with an enzyme preparation from *Patella vulgata* for the usual preliminary acid hydrolysis of the urine. Subsequently it was found that although 16-oxooestradiol-17β is stable to boiling for 60 min. with 15 vol. % of 10N-HCl in aqueous solution, it undergoes extensive destruction when subjected to the same treatment after solution in urine. It was also found that 16-oxooestradiol-17β undergoes extensive destruction when heated in ethanolic solution with acetic acid and Girard's Reagent T. It is concluded, therefore, that the amounts of 16-oxooestradiol-17β determined in the early experiments must have represented only small fractions of those that were actually present in the urine.

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The Isolation of a Ketonic Dihydroxy Kober Chromogen from the Urine of Pregnant Women

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(*Received* 3 *May* 1956)

In 1943 Pincus & Pearlman briefly referred in a review article to evidence obtained by them 'for the presence of an oestrogen in human pregnancy urine which is ketonic and contains a hydroxyl group other than the usual phenolic one'. Experimental details of this work were not published, but subsequently Huffman & Grollman (1947) suggested that this ketonic oestrogen might be 16-oxo-oestradiol-17β (oestra-1:3:5-triene-3:17β-diol-16-one), which previously had been prepared from oestrone by Huffman (1942) (cf. Huffman & Lott, 1948); it was also suggested by these authors that 16-oxooestradiol-17β might be an intermediate in the metabolic conversion of oestrone (oestra-1:3:5-trien-3-ol-17-one) into oestriol (oestra-1:3:5-triene-3:16α:17β-triol). More recently Migeon (1953) has reported the detection in extracts of acid-hydrolysed human urine of a H_2SO_4–fluorogen which behaved like 16-oxooestradiol-17β on counter-current distribution in two different solvent

systems; but since 16-oxooestradiol-17β added to urine could not be subsequently recovered after subjection of the latter to the usual acid hydrolysis and extraction procedures which were employed, it was concluded that the unidentified H_2SO_4–fluorogen was probably not identical with the former.

The suggestion that 16-oxooestradiol-17β might be an intermediate in the metabolism of the oestrogens was recently readvanced by Marrian & Bauld (1955) when they isolated 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) from the urine of pregnant women. Although, at that time, the possibility that the isolated substance was an artifact had not been excluded (cf. Watson & Marrian, 1956), it was nevertheless tentatively suggested that 16-oxooestradiol-17β might be the common metabolic precursor of oestriol and 16-*epi*oestriol.

Following up this suggestion Watson & Marrian (1955)* examined the urine of pregnant women for the presence of this hypothetical ketonic precursor of the two epimeric triols; they were able to detect a Kober chromogen in ether-soluble ketonic–phenolic fractions, which was more 'polar' than oestrone, and which was indistinguishable from 16-oxooestradiol-17β by chromatography in the system 70 % (v/v) methanol in water on Celite–25 % (v/v) ethylene dichloride in benzene, and by countercurrent distribution in the system 20 % (v/v) *n*-hexane in benzene–50 % (v/v) ethanol in water. In these experiments preliminary enzymic hydrolysis of the urine gave yields of the unknown Kober chromogen about 2–3 times higher than those obtained after conventional hot-acid hydrolysis; and since 16-oxooestradiol-17β was found to be labile to hot-acid treatment in urine this finding indicated a further similarity between the urinary Kober chromogen and this compound.

Although it was believed by Watson & Marrian (1955) that the unknown ketonic Kober chromogen (KC-5) was 16-oxooestradiol-17β, it was appreciated that the identity of the former could only be established with certainty after isolation. The work described in the present paper was accordingly undertaken.

RESULTS

Isolation of KC-5

Partition chromatographic analysis of ether-soluble ketonic–phenolic fractions obtained from batches of pooled late-pregnancy urine after hydrolysis with enzymic preparations from *Patella vulgata* showed these fractions to contain 3·4–4·4 % by weight of oestrone and 14–22 % by weight of KC-5 (calc. as oestriol) (Table 1). The most effective method of those tried for the further concentration of KC-5 in such fractions was found to be chromatography on a large Celite column with the system 70 % (v/v) methanol in water–20 % (v/v) *n*-hexane in benzene. By this method, for instance, a concentrate containing 82 % of KC-5 was obtained from batch III. The isolation of crystalline material from such concentrates was achieved by washing with a small volume of chilled methanol and then crystallizing from methanol at $-20°$. The yield of recrystallized material was poor, but further quantities of crystalline material were obtained from the methanolic washing and mother liquor by evaporation to dryness and re-treating with small volumes of chilled methanol.

The recrystallized KC-5 melted at 236·5–238·5°, and the melting point was not significantly depressed after admixture with 16-oxooestradiol-17β (m.p. 238–241°). It gave C and H analyses in excellent agreement with those required for a compound of the formula $C_{18}H_{22}O_3$. However, it was clear that KC-5 could not be identical with 16-oxooestradiol-17β since it had $[\alpha]_D^{15} + 143°$ in ethanol (cf. 16-oxooestradiol-17β, $[\alpha]_D^{12} - 89°$ in ethanol). Furthermore, in contrast to 16-oxooestradiol-17β, it developed its maximum colour in the Kober reaction with no heating in the first stage (Fig. 1),

Table 1. *Ketonic–phenolic fractions from enzyme-hydrolysed late-pregnancy urine: oestrone and KC-5 contents*

Batch	Vol. of urine (l.)	Wt. of ketonic–phenolic fraction (mg.)	Oestrone content of fraction (%)	KC-5 content (%)
I	75	207	3·5	15
II	101	377	3·5	14
III	100	284	3·4	22
IV	95	274	4·4	15

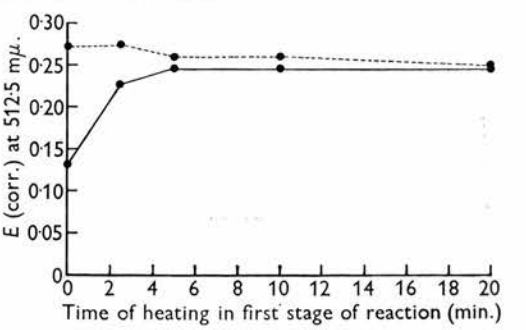

Fig. 1. Effect of variation in time of heating in the first stage of the Kober reaction on intensities of final colours developed by 16-oxooestradiol-17β (●—●) and KC-5 (●- - -●).

* The results obtained by Watson & Marrian (1955) were not reported in full detail. It is regretted that owing to limitations of space it is not possible to give these results in detail in the present paper.

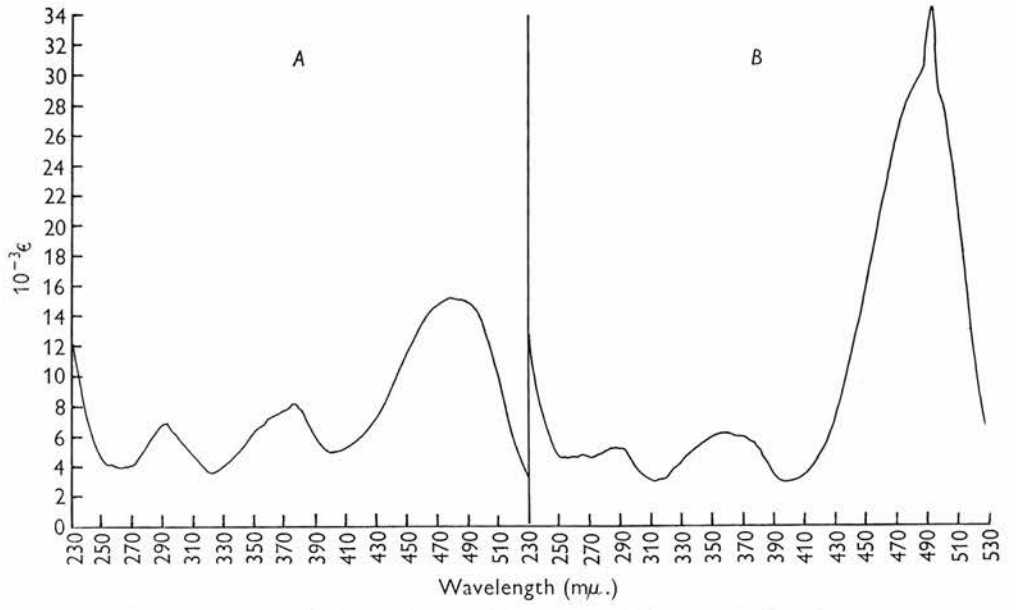

Fig. 2. Absorption spectra of H_2SO_4 solutions of 16-oxooestradiol-17β and KC-5 after 2 hr. at 24°. *A*, 16-Oxooestradiol-17β: $8{\cdot}39 \times 10^{-5}$ M; *B*, KC-5: $8{\cdot}74 \times 10^{-5}$ M.

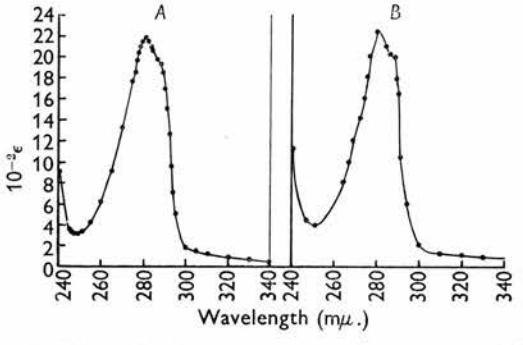

Fig. 3. Ultraviolet absorption spectra of 16-oxooestradiol-17β and KC-5 in ethanolic solution. *A*, 16-Oxooestradiol-17β: $1{\cdot}67 \times 10^{-4}$ M; *B*, KC-5, $1{\cdot}75 \times 10^{-4}$ M.

while the spectrum of its solution in conc. H_2SO_4 differed markedly from that of a similarly prepared solution of 16-oxooestradiol-17β (Fig. 2).

Characterization of KC-5

General. On acetylation KC-5 yielded a diacetate which melted at 166–167° (cf. 16-oxooestradiol-17β diacetate, m.p. 132·5–134°). This suggested the possibility that KC-5 might be 6-oxooestradiol-17β, the acetate of which melts at 173–175° (Longwell & Wintersteiner, 1940). However, this possibility was excluded by the ultraviolet-absorption spectrum in ethanol of KC-5, which was found to be virtually identical with those of 16-oxooestradiol-17β (Fig. 3), oestriol, 16-*epi*oestriol and oestrone, but markedly different from that of 6-oxooestradiol-17β as recorded by the above-mentioned authors.

In the 'blue tetrazolium' test carried out according to Mader & Buck (1952), KC-5 showed marked reducing power, suggesting that it was an α-ketol. However, the intensity of the colour developed in this test was only about one-third of that of the colour developed by an equivalent amount of 16-oxooestradiol-17β. This finding suggested the possibility that KC-5 might be a 16-hydroxyoestrone, since it had previously been found (Fotherby & Marrian, unpublished work) that 16α-hydroxyandrost-4-ene-3:17-dione (Fried, Thoma, Perlman, Herz & Borman, 1955) gives a considerably less intense colour in this test than those given by either 16-oxooestradiol-17β or pregn-4-ene-17α:21-diol-3:20-dione. The dextrorotation of KC-5 was consistent with the presence of a 17-oxo group (cf. Barton & Klyne, 1948), while the finding that the compound gave a negative Zimmermann reaction was consistent with the presence of 16-hydroxy-17-oxo grouping.

Sodium borohydride reduction of KC-5. To investigate the possibility of KC-5 being one or other of the two epimeric 16-hydroxyoestrones a sample of the isolated substance was reduced with sodium borohydride. Since by this method of reduction oestrone yields oestradiol-17β exclusively (Biel, 1951), it was expected that KC-5 would yield either oestriol or 16-*epi*oestriol, depending on whether the C-16 hydroxyl group of the former had the α- or β-configuration. Unfortunately, owing to the shortage

of available material this experiment had to be carried out on a sample of KC-5 which had been recovered from the mother liquors of the analytical sample.

On chromatographing a portion of the reduction product in the system 70% (v/v) methanol in water–ethylene dichloride the presence of two components in approximately equivalent amounts was unexpectedly revealed, and the elution characteristics of the less and more 'polar' of these were closely similar to those of 16-*epi*oestriol and oestriol respectively (Fig. 4). In view of this finding the main bulk of the reduction product was separated into its two components on a larger partition column with the same solvent system.

After recrystallization the less 'polar' component melted at 273–276°, and the melting point was not depressed after admixture with 16-*epi*oestriol (m.p. 284–285°). Because of the considerable difference in the melting points this was not accepted as completely satisfactory evidence of the identity of this reduction product with 16-*epi*-oestriol. However, on acetylation the reduction product yielded an acetate, m.p. 151–152°, and the mixed melting point of this with 16-*epi*oestriol triacetate (m.p. 151–152°) showed no depression. Accordingly, there could be no doubt that this reduction product was identical with 16-*epi*oestriol.

The more 'polar' reduction product of KC-5 melted at 277–279° after recrystallization, and the melting point was not depressed after admixture with oestriol (m.p. 279–280°). It yielded an acetate, m.p. 125–128°, and the mixed melting point with oestriol triacetate (m.p. 126–128°) showed no depression. Accordingly, this second reduction product of KC-5 was clearly identical with oestriol.

On the reasonable assumption that no inversion of either a C-16 or a C-17 hydroxyl group occurs during sodium borohydride reduction, the only α-ketol which could give rise to oestriol when reduced by this method is 16α-hydroxyoestrone. Accordingly, it can be concluded that the latter was present in the sample of KC-5 used in the reduction experiment.

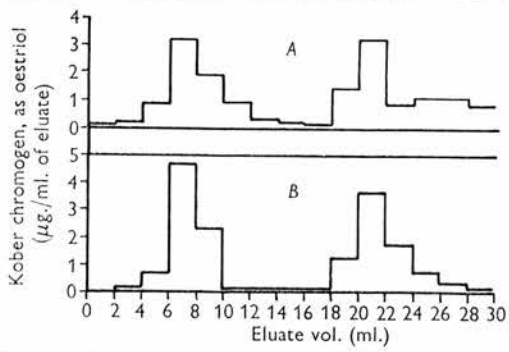

Fig. 4. Analytical column partition chromatograms (70% methanol in water–ethylene dichloride) on *A*, sodium borohydride reduction product from KC-5, and *B*, a mixture of 15·5 μg. of 16-*epi*oestriol and 16·3 μg. of oestriol.

On the other hand, either 16β-hydroxyoestrone or 16-oxooestradiol-17β could give rise to 16-*epi*-oestriol by sodium borohydride reduction, and, indeed, it has been shown by Huffman & Lott (1955) that 16-*epi*oestriol is formed in nearly quantitative yield when 16-oxooestradiol-17β is reduced by this procedure. It seems probable, therefore, that the sample of KC-5 used in the reduction experiment was a mixture either of 16α-hydroxyoestrone and 16β-hydroxyoestrone or of 16α-hydroxyoestrone and 16-oxooestradiol-17β, although the alternative explanation, that a partial rearrangement of 16α-hydroxyoestrone to 16-oxooestradiol-17β occurred during the reduction, cannot be excluded. As the sample of KC-5 used for the reduction was material recovered from the mother liquors of the analytical sample, it is not unlikely that it was grossly impure. The diacetate of KC-5, which was prepared from a mixture of recrystallized material and material recovered from the mother liquors, had a melting point 13° lower than that reported for synthetic 16α-hydroxyoestrone diacetate by Leeds, Fukushima & Gallagher (1954). This finding is consistent with the view that the KC-5 recovered from the mother liquors contained in addition to 16α-hydroxy-oestrone a considerable proportion either of 16β-hydroxyoestrone or of 16-oxooestradiol-17β.

While there is no reason to believe that the recrystallized KC-5 was a pure substance, it seems probable that the principal component in it must have been either 16α-hydroxyoestrone or 16β-hydroxyoestrone, and not the laevorotatory 16-oxo-oestradiol-17β. If the principal component in it was 16α-hydroxyoestrone, the accompanying impurity, which would presumably become concentrated in the mother liquor, could have been either 16β-hydroxyoestrone or 16-oxooestradiol-17β. On the other hand, if the principal component in it was 16β-hydroxyoestrone, the accompanying impurity must have been 16α-hydroxyoestrone.

Until a larger amount of more rigorously purified KC-5 has been examined a definite decision between these possibilities cannot be made. However, since Cooley, Ellis, Hartley & Petrov (1955) have shown that alkaline hydrolysis at room temperature of androst-5-ene-3β:16α-diol-17-one diacetate yields androst-5-ene-3β:17β-diol-16-one it is not improbable that if 16α-hydroxyoestrone had been present in the original urinary extracts the analogous rearrangement of 16α-hydroxyoestrone to 16-oxo-oestradiol-17β might have occurred to some extent during the extraction of the phenolic fraction with aqueous alkali. The view that the isolated KC-5 was

a mixture of 16α-hydroxyoestrone and artifactually produced 16-oxooestradiol-17β would seem to be entirely consistent with all the available evidence, and is therefore the favoured one.

EXPERIMENTAL

Methods

Enzymic hydrolysis of urine. Urine specimens were acidified to pH 4·7 with acetic acid, buffered to this pH by the addition of one-tenth of their volume of M acetate buffer, and incubated at 37° with the enzyme preparation for a period of 48 hr. In every instance half of the total amount of enzyme used was added initially and half after 24 hr.

Analytical partition chromatograms. The procedures based on those of Bauld (1955) and described by Marrian & Bauld (1955) and Watson & Marrian (1956) were employed.

Kober reaction. The method of Brown (1952), as modified by Bauld (1954), was employed, Bauld's 'oestriol reagent' being used. All determinations of Kober chromogens are expressed as oestriol equivalents.

Melting points. The melting points of 16-oxoestradiol-17β, KC-5, oestriol and 16-epioestriol were determined in sealed, evacuated capillary tubes, while for those of the acetates of these substances a microscope hot-stage was employed. The same thermometer was used for all determinations and the values given are uncorrected for emergent stem. Although corrected melting points were given for oestriol and 16-*epi*oestriol in a recent paper by two of the present authors (Watson & Marrian, 1956) we are now of the opinion that the method of correcting for emergent stem with the aid of pure standard compounds which was used is unreliable for temperatures much above 200°, because of the lack of reliable data for corrected melting points on suitable compounds.

Materials

16-*Oxooestradiol*-17β. This was prepared from oestrone by the method of Huffman (1942) and Huffman & Lott (1948), and was crystallized once from 50% (v/v) aqueous acetic acid and once from dilute methanol. The m.p. was 239–241° and it had $[\alpha]_D^{12}$ −89° (0·600% in ethanol). It yielded a diacetate, m.p. 132·5–134°.

Oestriol. The preparation described by Marrian & Bauld (1955) was used. The m.p. was 279–280° and it had $[\alpha]_D^{19}$ +61° (0·296% in ethanol). It yielded a triacetate, m.p. 126–128°.

16-epi*Oestriol.* The preparation described by Watson & Marrian (1956) was used. The m.p. was 284–285°, and it had $[\alpha]_D^{15}$ +76° (0·297% in ethanol). It yielded a triacetate, m.p. 151–153°.

Enzyme preparations. These were crude acetone-dried powders obtained from *Patella vulgata* by the method of Dodgson & Spencer (1953). They were standardized for β-glucuronidase activity with phenolphthalein glucuronide as substrate by the method of Fishman (1948). The sulphatase activities of these preparations were not determined.

Miscellaneous. The solvents and Celite (Celite 535, Johns Manville and Co., Ltd., London) used were purified by the same methods employed by Watson & Marrian (1956).

Isolation of KC-5 from pregnancy urine

Late-pregnancy urine was enzymically hydrolysed in batches of 8–16 l. with amounts of the enzyme equivalent to about 1 000 000 Fishman units/l. of urine. The hydrolysed mixture was extracted once with an equal volume of ether after the addition of 600 g. of NaCl/4 l. of urine, the extract washed once with one-tenth of its volume of 5% (w/v) $NaHCO_3$ and then extracted twice with one-eighth volume of N-NaOH. The NaOH extract was partly neutralized by the addition of 5N-H_2SO_4, and then neutralization (to phenolphthalein) completed by passing in CO_2. The extraction with NaOH and the subsequent neutralization of the extract were carried out as rapidly as possible to minimize losses of KC-5 which occur in strongly alkaline solutions.

The neutralized extract was extracted twice with equal volumes of ether, the extract washed twice with one-quarter volumes of water, dried over $NaSO_4$ and evaporated to dryness.

Phenolic fractions obtained in this way were stored at 0° until 100 l. of urine had been processed. They were then combined and treated at room temperature for 17 hr. with 5 g. of trimethylammonium hydrazide chloride in 50 ml. of ethanol and 10 ml. of acetic acid. The ketonic–phenolic fraction obtained from the reaction mixture in the usual way weighed 284 mg., and by carrying out an analytical partition chromatogram on one-thousandth of the total amount it was shown to contain 9·7 mg. of oestrone and 63·4 mg. of KC-5.

The main bulk of the phenolic–ketonic fraction was chromatographed on a large column (2 cm. diam.) with 120 g. of Celite and the solvent system 70% (v/v) methanol in water–20% (v/v) *n*-hexane in benzene. Thirty successive 40 ml. fractions of the eluate were collected and evaporated to dryness, and Kober reactions carried out on small portions of each. Fractions 19–22, which contained the KC-5, were combined, yielding 55·3 mg. of a slightly pigmented solid which was found to contain 45·5 mg. of Kober chromogen. By washing twice with methanol chilled to −20° a white solid (35·0 mg.) was obtained. After this had been combined with 15·7 mg. of similarly prepared material from another batch of urine it was recrystallized from methanol at −20°, yielding 9·2 mg. of 'Grade I KC-5'. After drying for 2 hr. at 100° *in vacuo* the product had: m.p. 236·5–238·5°; $[\alpha]_D^{15}$, +143° (*c*, 0·254% in ethanol); ϵ_{286}, 2238 in ethanol (Fig. 3). (Found: C, 75·5; H, 7·9. Calc. for $C_{18}H_{22}O_3$: C, 75·5; H, 7·8%.)

By further treatment of the material obtained by evaporation of the methanol washings and mother liquors by rewashing with chilled methanol, 24·6 mg. of 'Grade II KC-5', m.p. 235–238°, and 15·3 mg. of 'Grade III KC-5', m.p. 234–238°, were obtained.

Properties and characterization of KC-5

Kober reaction on KC-5 and 16-oxooestradiol-17β: variation in time of heating in first stage of reaction. The technique used in this experiment was the same as that described by Marrian & Bauld (1955) for a similar experiment with 16-*epi*oestriol (KC-4) and oestriol. For the Kober reactions on KC-5, 10 μg. of the latter (Grade I) was used in each tube, and, for those on 16-oxooestradiol-17β, 11 μg. was used. The curves showing the relationship between optical density at 512·5 mμ. [corrected by using the Allen (1950) formula] are shown in Fig. 2.

Reaction with conc. H_2SO_4 *on KC-5 and 16-oxooestradiol-17β.* To each of two tubes containing respectively 100 μg. of KC-5 (grade I) and 96 μg. of 16-oxooestradiol-17β was added 4 ml. of conc. H_2SO_4, and the mixtures were allowed

to remain at 24° for 2 hr. with occasional shaking. Absorption spectra over the range 230–530 mμ. were determined on both solutions (Fig. 3). It is noteworthy that on adding the H_2SO_4 to the KC-5 an intense red colour developed immediately. This was not observed with 16-oxooestradiol-17β.

KC-5 diacetate. KC-5 (6·7 mg. of grade I + 8·4 mg. of grade III) was treated at room temperature for 24 hr. with 0·5 ml. of acetic anhydride and 0·5 ml. of anhydrous pyridine. After the addition of ice and water, the crude acetate was filtered off, washed thoroughly with water, dried, and crystallized once from a mixture of ethyl acetate and *n*-hexane. The product melted at 166–167°, and the mixed m.p. with 16-oxooestradiol-17β diacetate (m.p. 132·5–134°) was 120–142°. After drying for 1 hr. at 80° *in vacuo* the product had $[\alpha]_D^{13}$, +156° (*c*, 0·301% in ethanol). (Found: C, 71·2; H, 6·8. Calc. for $C_{22}H_{26}O_5$: C, 71·3; H, 7·1%.)

Sodium borohydride reduction of KC-5. To a solution of 20 mg. of KC-5 (Grade II) in 10 ml. of methanol was added 15 mg. of sodium borohydride. After standing at room temperature for 40 min. a further 10 mg. of sodium borohydride was added and the mixture allowed to stand for a further 60 min. About half of the methanol was evaporated off under reduced pressure in a warm-water bath, and after dilution with 100 ml. of water and acidification with 4 ml. of 10 N-HCl the mixture was extracted thoroughly with ether. The ethereal extract was washed with aqueous $NaHCO_3$ and with water, and evaporated to dryness. The product was treated at room temperature for 17 hr. with 250 mg. of trimethylammonium hydrazide chloride in 5 ml. of ethanol and 5 ml. of acetic acid, and the non-ketonic material was obtained in the usual way by ether extraction after dilution with water and partial neutralization with NaOH. The non-ketonic reduction product weighed 20·2 mg.

One five-hundredth of the product was chromatographed in the system 70% (v/v) methanol in water–ethylene dichloride, and the results indicated the presence in the total product of about 7·9 mg. of a less 'polar' Kober chromogen and about 9·6 mg. of a more 'polar' one. For comparison a mixture of 15·5 μg. of 16-*epi*oestriol and 16·3 μg. of oestriol were chromatographed in the same system (Fig. 4).

The main bulk of the non-ketonic reduction product was separated into its two components on a large partition column with 120 g. of Celite and the same solvent system. The less and more 'polar' fractions obtained weighed 6·4 and 7·8 mg. respectively.

After one crystallization from methanol–benzene the less 'polar' product melted at 273–276°, and the mixed m.p. with 16-*epi*oestriol (m.p. 284–285°) was 274–281°. The remainder of the recrystallized material, together with that obtained by evaporation of the mother liquor, was acetylated in the usual way with acetic anhydride and pyridine at room temperature. The product, after one crystallization from *n*-hexane, melted at 151–152° and the mixed m.p. with 16-*epi*oestriol triacetate (m.p. 151–153°) was 150–152°. The identification of the less 'polar' reduction product of KC-5 as 16-*epi*oestriol was therefore considered to be satisfactory.

The more 'polar' reduction product melted at 277–279° after one crystallization from methanol–benzene, and the mixed m.p. with oestriol (m.p. 279–280°) was 277–280°.

DISCUSSION

The average yield of KC-5 found in pooled late-pregnancy urine, as determined by analytical partition chromatograms on the ketonic–phenolic fractions, was 0·5 mg./l.; and, since the extraction and fractionation processes employed were certainly not quantitative, it is probable that the average concentration of KC-5 actually present in urine is considerably higher. It is clear, therefore, that this newly discovered α-ketolic Kober chromogen is an oestrogen metabolite of considerable quantitative importance. Although it is present in late-pregnancy urine in such comparatively large amounts, it is hardly surprising, in view of its considerable lability to certain of the processes commonly employed in urine extraction and fractionation, that it has not hitherto been isolated. Rather it is surprising that it was detected in urine extracts by Watson & Marrian (1955), and also, presumably by Pincus & Pearlman (1943) and by Migeon (1953), in experiments in which conditions for its survival must have been far from optimum.

Although it is probable that the material isolated was not a pure substance, convincing evidence has been obtained which shows that the principal component in it must have been either 16α-hydroxyoestrone or 16β-hydroxyoestrone. While other evidence, which has already been discussed, is consistent with the view that the isolated KC-5 was composed of 16α-hydroxyoestrone and some artifactually produced 16-oxooestradiol-17β, the results of the present investigation indicate that 16-oxooestradiol-17β is probably not present as such in urine. Accordingly the speculative suggestion advanced by Marrian & Bauld (1955) that 16-oxooestradiol-17β might be the common metabolic precursor of oestriol and 16-*epi*oestriol receives no support.

The view that the principal component in KC-5 is 16α-hydroxyoestrone rather than 16β-hydroxyoestrone can be supported on biogenetic grounds. It now seems reasonable to suppose that both of these compounds may be formed metabolically from oestrone by 16α- and 16β-hydroxylation, and that they are intermediates in the 'hydration' of oestrone to oestriol and 16-*epi*oestriol respectively. Since the concentration of oestriol in late-pregnancy urine is about 60 times that of 16-*epi*oestriol, it might be assumed that the concentration of the immediate precursor of oestriol would be much greater than that of the immediate precursor of 16-*epi*oestriol.

SUMMARY

1. An α-ketolic Kober chromogen (KC-5) has been isolated from the urine of pregnant women.

2. The evidence obtained shows that the principal component in KC-5 was either 16α-hydroxyoestrone or 16β-hydroxyoestrone. Reasons for favouring the first alternative are given.

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